

**THE STRUCTURE AND FUNCTION OF  
PERTUSSIS TOXIN**

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**TO MY MUM AND DAD**

## PREFACE

The work reported in this thesis was carried out between 1st October 1986 and 30th September 1989 under the supervision of Dr. Simon van Heyningen at the Department of Biochemistry, University of Edinburgh Medical School, or under the supervision of Dr. Andrew Robinson and Dr Lawrence Irons at PHLS Centre for Applied Microbiology and Research, Porton, Wiltshire. Unless stated otherwise, all work presented is the sole work of the author, as is the composition.

PHLS - Public Health Laboratory Service

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PAGE ORDER INACCURATE IN ORIGINAL

## ABBREVIATIONS

<b>A1</b>	=	The A1 chain of cholera toxin
<b>AC</b>	=	adenylate cyclase
<b>ADP</b>	=	adenosine diphosphate
<b>ADP-ribose</b>	=	adenosine diphosphoribose
<b>AGG's</b>	=	agglutinogens
<b>AMP</b>	=	adenosine monophosphate
<b>cAMP</b>	=	cyclic adenosine monophosphate
<b>ATP</b>	=	adenosine triphosphate
<b>A599</b>	=	[12-(phenyl methyl amino)dodecanyl] guanidine
<b>A603</b>	=	[10-(acetyl amino)decanyl] guanidine
<b>BSA</b>	=	bovine serum albumin
<b>CaM</b>	=	calmodulin
<b>CAMR</b>	=	Centre for Applied Microbiology and Research
<b>CD</b>	=	circular dichroism
<b>CHAPS</b>	=	3-[(3-cholamidopropyl)dimethylammonia]propane sulfonate
<b>CL+CD</b>	=	medium of Imaizumi et al., (1983) plus Me CD
<b>CHO</b>	=	Chinese hamster ovary
<b>Cys</b>	=	cysteine
<b>DG</b>	=	diacyl glycerol
<b>DSP</b>	=	dithiobis (succinimidyl propionate)
<b>DST</b>	=	disuccinimidyl tartarate
<b>DTT</b>	=	dithiothreitol
<b>DMSO</b>	=	dimethyl sulphoxide
<b><i>E. coli</i></b>	=	<i>Escherichia coli</i>
<b>EDTA</b>	=	ethylene diamine tetra acetic acid

<b>EF-2</b>	=	elongation factor 2
<b>ELISA</b>	=	enzyme linked immunosorbant assay
<b>FHA</b>	=	filamentous haemagglutinin
<b>FPLC</b>	=	fast protein liquid chromatography
<b>GABA</b>	=	gamma aminobutyric acid
<b>GlcNAc</b>	=	N-acetyl glucosamine
<b>GMP</b>	=	guanosine monophosphate
<b>cGMP</b>	=	cyclic guanosine monophosphate
<b>GMI</b>	=	galactosyl-N-acetyl glucosamine-[N acetyl neuraminyl]- galactosylglucosyl-ceramide
<b>Gi</b>	=	inhibitory regulatory unit of adenylate cyclase
<b>Gs</b>	=	stimulatory regulatory unit of adenylate cyclase
<b>Gproteins</b>	=	GTP-binding proteins
<b>GTP</b>	=	guanosine monophosphate
<b>HLT</b>	=	heat-labile toxin
<b>HPLC</b>	=	high pressure liquid chromatography
<b>KDO</b>	=	3-deoxy-2 octulosonic acid
<b>kcat</b>	=	catalytic capacity
<b>Kd</b>	=	dissociation constant
<b>Km</b>	=	Michaelis constant
<b>LPS</b>	=	lipopolysaccharide endotoxin
<b>MeCD</b>	=	2,6-O dimethyl $\beta$ -cyclodextrin
<b>NAD<sup>+</sup></b>	=	nicotinamide adenine dinucleotide

Neu Ac	=	N-acetyl neuraminic acid
Neu Ac Gal $\beta$ -Glc N Ac	=	N-acetyl neuramic acid galactose $\beta$ -4 N-acetyl glucosamine
OD	=	optical density
Pi P <sub>2</sub>	=	phosphatidyl inositol (4,5) biphosphate
PBS	=	phosphate buffered saline
PHLS	=	Public Health Laboratory Service
Ri	=	inhibitory receptor of adenylate cyclase
Rs	=	stimulatory receptor of adenylate cyclase
SDS PAGE	=	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCT	=	trachael cytotoxin
Trp	=	tryptophan
Vmax	=	maximum catalytic capacity of the enzyme

## ABSTRACT

Pertussis toxin is the most complex of the bacterial toxins discovered to date. It has an estimated molecular weight of 117000 and catalyses the ADP-ribosylation of a regulatory protein of adenylate cyclase,  $G_i$ , preventing hormonal inhibition of cyclase activity resulting in a build-up of intracellular cAMP levels. As  $G_i$  is notoriously difficult to isolate and very labile, the toxin catalysed  $NAD^+$ -glycohydrolase is routinely used as a measure of its activity. A  $K_m$  of  $27\mu M$  for  $NAD^+$  was obtained for this reaction. The use of reducing reagents is essential within this assay procedure as reduction of an internal disulphide bond is required for enzymic activity. 250mM reducing reagent resulted in maximum activity, a concentration which appeared very high if the reagent is only required for activation. The sulphhydryl containing reagent were shown to be both essential for enzymic activity and were also substrates for the reaction. An ADP-ribosyl-cysteine reaction product has been isolated. A Michaelis constant of 105mM was obtained for cysteine acting as a substrate in saturating  $NAD^+$ .

Binding of  $NAD^+$  to pertussis toxin was investigated by both equilibrium dialysis and quenching of the toxins intrinsic fluorescence on titration with  $NAD^+$ . Equilibrium dissociation constants,  $K_d$  values, were obtained at a number of different temperatures by equilibrium dialysis and a value of  $57kJoules\ mol^{-1}$  was derived for the enthalpy change on binding of substrate to protein, a value typical of such an interaction. The number of binding sites on the toxin for  $NAD^+$  was shown to be one. Equilibrium dialysis was carried out in a urea solution in order to obtain sufficiently high protein concentrations to minimise error. Urea, at the concentration used, was shown to have no effect on the tertiary structure of the toxin by circular dichroism and as such should not affect the binding of ligand to protein. The results from equilibrium dialysis and fluorescence were in close agreement and correlated well with the  $K_m$  value obtained for the toxin catalysed  $NAD^+$ -glycohydrolase activity.

The association of the toxin subunits in space and its overall stoichiometry were

investigated using reversible cross-linking reagents, two-dimensional gel electrophoresis and Western blotting. The results obtained suggest that S-4 is in close association with subunits S-2 and S-3, providing further evidence for the existence of two dimers within the holotoxin, and show that S-1 is within close proximity of subunits S-2 and S-3. The latter suggests that S-1 is on a plane above the B-digomer subunits, in the same way as has been proposed for cholera toxin. No cross-links have so far been obtained involving S-5 indicating that the other subunits may surround this connecting peptide in such a way as to shield it from the cross-linkers.

## CONTENTS

	PAGE
<b>CHAPTER 1      INTRODUCTION</b>	
1.1.      Pertussis the disease	1
1.1.2      Bordetella pertussis	2
1.1.3      Treatment and control	3
<b>1.2      Biologically active substances produced by           <i>B. pertussis</i></b>	<b>4</b>
1.2.1      Agglutinogens	4
1.2.2      Filamentous haemagglutinin	6
1.2.3      Adenylate cyclase	7
1.2.4      Lipopolysaccharide endotoxin	10
1.2.5      Dermonecrotizing (heat-labile) toxin	12
1.2.6      Tracheal cytotoxin	12
<b>1.3      The structure and function of pertussis toxin</b>	<b>14</b>
1.3.1      Introduction	14
1.3.2      The structure of pertussis toxin	16
1.3.3      Molecular cloning of pertussis toxin genes and its primary structure	20
1.3.4      The role of each subunit	23
1.3.5      The interaction of pertussis toxin with the cell membrane	23
1.3.6      The entry of toxin into the cell	24
1.3.7      ADP-ribosylation and activation of adenylate cyclase	26
1.3.8      Other protein substrates for pertussis toxin	29
<b>1.4      Other toxins</b>	<b>33</b>
1.4.1      Cholera toxin	33
1.4.2 <i>E. coli</i> heat labile toxin	34

1.4.3	Diphtheria toxin	35
1.4.4	<i>Pseudomonas aeruginosa</i> exotoxin A1	35
1.4.5	The cytotoxins of <i>Clostridium botulinum</i>	36
1.4.6	The neurotoxins of <i>Clostridium botulinum</i>	37
1.4.7	Tetanus toxin	37
<b>CHAPTER 2</b>	<b>MATERIALS AND METHODS</b>	<b>39</b>
<b>2.1</b>	<b>Materials</b>	<b>39</b>
<b>2.2</b>	<b>Methods</b>	<b>40</b>
2.2.1	Preparation of pertussis toxin - general method	40
2.2.2	Electrophoretic techniques	42
2.2.2.1	SDS polyacrylamide gel electrophoresis (PAGE) general method	42
2.2.2.2	Gradient polyacrylamide gel electrophoresis	43
2.2.2.3	Western blotting	43
2.2.2.4	Silver staining	44
2.2.2.5	FPLC separation of pertussis toxin subunits	44
<b>2.2.3</b>	<b>NAD<sup>+</sup> studies</b>	<b>45</b>
2.2.3.1	Assay of NAD <sup>+</sup> -glycohydrolase - general method	45
2.2.3.2	Activation of pertussis toxin	45
2.2.3.3	Determination of the efficiency of NAD <sup>+</sup> -glycohydrolase	46
2.2.3.4	Sulphydryl reagents as potential substrates	46
2.2.3.5	HPLC separation of NAD <sup>+</sup> -glycohydrolase reaction products	47
2.2.3.6	Identification of reaction products using radioactive compounds	47
2.2.3.7	Isolation of putative ADP-ribosyl-cysteine	48
2.2.3.8	Reaction of snake-venom phosphodiesterase	48
2.2.3.9	Equilibrium dialysis	48
2.2.3.10	Fluorescence measurements	49



2.2.3.11	Circular dichroism of pertussis toxin	49
<b>2.2.4</b>	<b>Cross-linking studies</b>	50
2.2.4.1	Cross-linking of pertussis toxin subunits	50
2.2.4.2	Two-dimensional SDS-polyacrylamide gel electrophoresis	50
2.2.4.3	Electroelution	51
<b>2.2.5</b>	<b>Determination of protein</b>	52
<b>2.2.6</b>	<b>Liquid scintillation counting</b>	52
<b>CHAPTER 3</b>	<b>GROWTH OF <i>BORDETELLA PERTUSSIS</i> AND PURIFICATION OF PERTUSSIS TOXIN</b>	53
<b>3.1</b>	<b>Introduction</b>	53
3.1.1	Expression of pertussis toxin	53
3.1.2	Growth of <i>Bordetella pertussis</i>	53
3.1.3	Growth of <i>Bordetella pertussis</i>	54
3.1.3.1	Initiation of growth	54
3.1.3.2	Growth medium	54
3.1.3.3	Preparation of CL and CD medium	55
<b>3.2</b>	<b>Purification of pertussis toxin</b>	56
3.2.1	Introduction	56
3.2.2	Purification of pertussis toxin	57
3.2.2.1	Hydroxyl apatite chromatography	57
3.2.2.2	Preparation of fetuin-Sepharose 4B	58
3.2.2.3	Fetuin-Sepharose 4B affinity chromatography	59
<b>3.3</b>	<b>Results</b>	59
3.3.1	Growth of <i>Bordetella pertussis</i>	59
3.3.2	Haemagglutinin test	60
3.3.2.1	Preparation of goose red blood cells	60
3.3.2.2	Haemagglutinin test	61

<b>3.4</b>	<b>Isolation of pertussis toxin subunits</b>	<b>63</b>
<b>3.5</b>	<b>Discussion</b>	<b>65</b>
3.5.1	Purification of pertussis toxin	65
3.5.2	Isolation of subunits	66
<b>CHAPTER 4</b>	<b>KINETIC ANALYSIS OF PERTUSSIS TOXIN</b>	<b>67</b>
4.1.1	Introduction	67
4.1.2	Kinetic models	70
<b>4.2</b>	<b>Results</b>	<b>71</b>
4.2.1	Characterisation of NAD <sup>+</sup> -glycohydrolase assay	71
4.2.2	Controls of NAD <sup>+</sup> -breakdown	74
4.2.3	Kinetics of NAD <sup>+</sup> -glycohydrolase reaction	76
4.2.4	Kinetics of NAD <sup>+</sup> -glycohydrolase reaction with sulphydryl group as substrate	80
4.2.5	Isolation of an ADP-ribosyl-sulphydryl compound	81
4.2.6	Treatment with phosphodiesterase	91
4.2.7	Investigation of other putative substrates	97
<b>4.3</b>	<b>Discussion</b>	<b>97</b>
<b>CHAPTER 5</b>	<b>BINDING OF NAD<sup>+</sup> TO PERTUSSIS TOXIN</b>	<b>105</b>
<b>5.1</b>	<b>Introduction</b>	<b>105</b>
<b>5.2</b>	<b>Results</b>	<b>107</b>
5.2.1	The principles of equilibrium dialysis	107
5.2.2	Dialysis time	108
5.2.3	Recovery of radioactivity	110
5.2.4	Non-specific breakdown of NAD <sup>+</sup>	110
5.2.5	Binding experiments	110
5.2.6	Quenching of protein fluorescence by NAD <sup>+</sup>	121

5.2.7	Fluorescence results	124
5.2.8	Circular dichroism	125
<b>5.3</b>	<b>Discussion</b>	132
5.3.1	Equilibrium dialysis	138
5.3.2	Calculation of constants	139
5.3.3	Intrinsic fluorescence	139
5.3.4	The nature of the NAD <sup>+</sup> -binding site	140
<b>CHAPTER 6</b>	<b>CROSS-LINKING OF PERTUSSIS TOXIN SUBUNITS</b>	146
<b>6.1</b>	<b>Introduction</b>	146
<b>6.2.</b>	<b>Cross-linking</b>	147
<b>6.3</b>	<b>Two-dimensional SDS polyacrylamide gel electrophoresis</b>	148
<b>6.4</b>	<b>Results</b>	150
6.4.1	Two dimensional SDS polyacrylamide gels	150
6.4.2	Western blotting	158
<b>6.5</b>	<b>Discussion</b>	160
6.5.1	Cross-linking and two-dimensional SDS polyacrylamide gele electrophoresis	160
6.5.2	Western blotting	161
<b>CHAPTER 7</b>	<b>RESULTS AND FUTURE WORK</b>	164
<b>REFERENCES</b>		167
<b>APPENDIX</b>	Further binding data	181

## CHAPTER 1

### INTRODUCTION

#### 1.1 Pertussis the disease

Pertussis, or as it is more commonly known "whooping cough", is an acute disease of the human respiratory tract, the symptoms of which are most severe in young children. The disease has been clinically recognised since the 16th century, with the first published account of symptoms being in 1640. Pertussis was a major cause of childhood morbidity and mortality until the gradual introduction of mass immunisation from the late 1940's, with most children being immunised from about 1957. This vaccination programme dramatically reduced the incidents of whooping cough in the following decades, but recently, owing to the much publicised possibility of side-effects, uptake rates of this vaccine have decreased because of a decline in the public's confidence with the vaccine to it. This has led to two recent epidemics of the disease in the U.K., one in 1979 and the other in 1982. The acceptance rate is now back to about 70% and the disease is less common, but the constant threat of another epidemic illustrates the need for a new vaccine which would hopefully reduce the possibility of side effects (Robinson *et al.*, 1985).

Pertussis exists worldwide and is still today a major problem in Third World countries where  $0.5-1 \times 10^6$  cases are reported each year. The disease is transmitted by droplet infection and there is a cyclical pattern of disease occurring every three to five years (Fine and Clarkson, 1982) as is also the case with the diseases measles and rubella.

The disease can be sub-divided into three symptomatic stages (Friedman, 1988), these being the catarrhal, paroxysmal and convalescent stages. The bacteria must first colonise the ciliated epithelium of the upper respiratory tract and this initial incubation period can last from six to twenty days (Manclark, 1976). Once the host tissue has been colonised the symptoms of the catarrhal stage manifest. These are similar to a mild cold with some associated inflammation of the nose and throat mucous membranes. As this stage of the disease progresses the patient begins to

suffer bouts of coughing which increase in severity with time, the bouts becoming more spasmodic as the disease enters the paroxysmal stage. Initially coughing is more frequent at night, but as the final stage of the disease progresses the bouts are more prevalent during the day. A child may suffer fifteen to twenty five bouts of paroxysmal coughing each day at the height of the disease (Manclark and Cowell, 1984). The child must concentrate all of its energy on clearing the air-passages of mucous and with each successive coughing bout the effects become more violent. The characteristic "whoop" occurs as a result of the child finally clearing the lungs of mucous and rapidly inspiring air into the lungs through the swollen glottis. Pertussis, meaning severe cough, is a much better term for describing the disease as the patient does not whoop with every bout of coughing (Wilson et al., 1965; Zoumboulakis et al., 1973).

Many complications can occur during this acute stage of the disease, including conjunctival haemorrhage, haemorrhage of the neck and shoulders, herniae, emphysema, prolapsed rectum and pneumothorax. The most dangerous complications, however, are associated with secondary bacterial infections. The mortality rate of pertussis is greatest in children less than one year old with the major cause of death being as a consequence of secondary infection (Miller and Fletcher, 1976).

#### 1.1.2. *Bordetella pertussis*

The bacterium responsible for the disease pertussis is *Bordetella pertussis*. The bacteria are gram-negative cocobacilli and were first isolated in 1906 by Bordet and Gengou from an infected patient (Bordet and Gengou, 1906). The organism is a human pathogen and is found only on the ciliated epithelium of the human upper respiratory tract (Mallory and Horner, 1912). There are four species in the genus *Bordetella*; *Bordetella pertussis* and *Bordetella parapertussis* which are both human pathogens, *Bordetella bronchiseptica* which is an animal pathogen and *Bordetella avium* which infects birds. All four are non-invasive respiratory tract parasites which share certain characteristics and cause similar disease in their host species.

*Bordetella pertussis* produces many biologically active substances, some of which may play a part in the disease process. However, the precise role of each of these virulence factors in the pathogenesis of disease is still under a great deal of investigation (Munoz and Bergman, 1979; Wardlaw and Parton, 1983). One of these proteins, pertussis toxin, which is the protein of interest in this project, may be the major virulence factor produced by the bacteria (Pittman, 1979; Sekura et al., 1983; Nicosia and Rappuoli, 1987; Gross et al., 1989). Whether all of the symptoms of pertussis result from the activity of this single exotoxin is a matter of much controversy.

### 1.1.3. Treatment and control

*B. pertussis* is sensitive to several antibiotics, but their use is limited after the onset of the catarrhal stage of the disease. These antibiotics include erythromycin, chloramphenicol and tetracycline, with chloramphenicol being the antibiotic generally used in treatment as it is both safe and effective to use in children (Bass et al., 1969; Islur et al., 1975). The treatment of pertussis with antibiotics has no effect upon the course of the disease, but they may reduce the time during which the host is infectious and may also help in the treatment of secondary bacterial infections (Baraff et al., 1978).

The vaccines currently used for the prevention of pertussis in most countries are suspensions of killed, whole-cells of *B. pertussis* (Robinson et al., 1985). These vaccines have been the cause of much public concern due to the rare number (1 in 100,000 at most) of associated cases of brain damage. However, it is true to say that the advantages of pertussis vaccination are far greater than any of the risks associated with it. In France, for example, where a vaccine of similar composition to that used in the U.K. is administered, there is very little debate on the risk of pertussis vaccination. It is estimated that 95% of children are immunised against whooping cough and this high vaccine uptake rate is reflected in the lack of a recent epidemic of whooping cough in this country (Miller et al., 1982).

The major concern of pertussis research in recent years has been the

development of a new acellular vaccine. Acellular vaccines have been developed in Japan and have been in use there for several years. The current, or shortly available vaccines range from pure detoxified pertussis toxin (Sekura *et al.*, 1986) through filamentous haemagglutinin/pertussis toxin vaccines (Sato *et al.*, 1984) to a filamentous haemagglutinin-pertussis toxin - agglutigen vaccine (Robinson *et al.*, 1985), with each of these vaccine constituents being detoxified proteins produced by *B. pertussis*. In view of this a great deal of work has concentrated on the purification and characterisation of the biologically active substances produced by the bacteria.

The next stage in acellular vaccine production should involve the isolation of subunits, peptide fragments or the preparation of synthetic peptides which contain epitopes essential to induce a protective immune response in children (Robinson and Ashworth, 1988). Much research is presently concentrated on production of the above with the continued aim of producing vaccine components which are immunogenic but non-toxic.

## **1.2. Biological active substances produced by *B. pertussis***

### **1.2.1. Agglutinogens**

There are a total of fourteen agglutinogens produced by all species within the genus *Bordetella*, with *B. pertussis* expressing seven of them in various combinations on different strains. Agglutinogens are surface antigens which can stimulate the production of antibodies which in turn cause bacterial cell agglutination (Robinson *et al.*, 1985). They are proteins of molecular masses ranging within 10 000 - 23 000. AGG's 1, 2 and 3 are the major agglutinogens and 4, 5 and 6 are minor antigens. The biochemical nature of the agglutinogens are summarised in Table. 1.1.

AGG 1 occurs on all strains of *B. pertussis*. The nature of this protein has not been determined, but it does not appear to be a fimbrial protein. AGG's 2 and 3 are fimbrial proteins of molecular mass 22 500 (Livey *et al.*, 1987) and 22 000 (Amelina *et al.*, 1976) respectively. The amino acid sequences of AGG's 2 and 3 have been determined and they exhibit 60% sequence homology. AGG's 4, 5 and 6 are minor

antigens and are apparently associated with either AGG 2 or 3.

**TABLE 1.1**      **Agglutinogens of *Bordetella pertussis***

<b>Agglutinin</b>	<b>Species Specificity</b>	<b>Serotype Specificity</b>	<b>Biochemical Structure</b>
<b>1</b>	<b><i>B. pertussis</i></b>	<b>All strains</b>	<b>Unknown Outer-membrane protein?</b>
<b>2</b>	<b><i>B. pertussis</i></b>	<b>1.2.0; 1.2.3</b>	<b>Fimbrial protein 22.5kDa subunit</b>
<b>3</b>	<b><i>B. pertussis</i></b>	<b>1.0.3; 1.2.3</b>	<b>Fimbrial protein 22 kDa subunit</b>
<b>4</b>	<b><i>B. pertussis</i></b>	<b>Minor associated with AGG2</b>	<b>Unknown</b>
<b>5</b>	<b><i>B. pertussis</i></b>	<b>Minor associated with AGG2</b>	<b>Unknown</b>
<b>6</b>	<b><i>B. pertussis</i></b>	<b>Minor associated with AGG3</b>	<b>Unknown</b>
<b>7</b>	<b><i>B. pertussis</i></b>	<b>All strains</b>	<b>Unknown</b>



It has been proposed by Robinson et al., (1989) that these minor structures may represent minor epitopes in association with the main fimbrial structural subunits, or, on the other hand, that they may perhaps be minor fimbrial proteins other than these repeating structural subunits.

The importance of the agglutinogens in pertussis immunity was demonstrated by the MRC clinical trials of whole-cell pertussis vaccines when the agglutinin response to vaccines was shown to correlate with their protection in children (MRC Report, 1959). The CAMR acellular vaccine consists of equal amounts of separately purified pertussis toxin, filamentous haemagglutinin and AGG's 2 and 3 (10µg/dose). Each component is separately formaldehyde treated and then combined and adsorbed on an aluminium adjuvant (Robinson et al., 1986). The AGG's are isolated from cells by homogenisation and repeated ammonium sulphate precipitation with AGG's 2 and 3 being present in the ratio of 3-2 respectively within the vaccine.

### 1.2.2. Filamentous haemagglutinin

Filamentous haemagglutinin (FHA) is a protein which has been shown by electron microscopy to be a filamentous structure of 2nm in diameter and 40-100nm in length (Arai and Sato, 1976; Morse and Morse, 1976). It was originally thought that FHA was derived from bacterial surface fimbria (Sato et al., 1979) and was known as filamentous and fimbrial-haemagglutinin. Recent evidence has shown this not to be true as both the amount of FHA did not correlate with the number of fimbriae and that fimbriae could not be labelled with antibodies of FHA (Ashworth et al., 1982).

Purified FHA is a heterogeneous mixture of polypeptides of Mr 90 000 - 220 000 (Irons et al., 1983). The degree of heterogeneity is dependent on the strain used and the growth conditions. Filamentous haemagglutinin is one of the two haemagglutinins secreted by *B. pertussis*, pertussis toxin being the other. Both of these proteins have very distinct chemical and biological properties. Antibodies against FHA inhibit the adhesion of *B. pertussis* to mammalian cells (Sato et al., 1974; Sato et al., 1979) demonstrating that this protein must be important in *sticking* the bacteria to host cells.

### 1.2.3. Adenylate cyclase

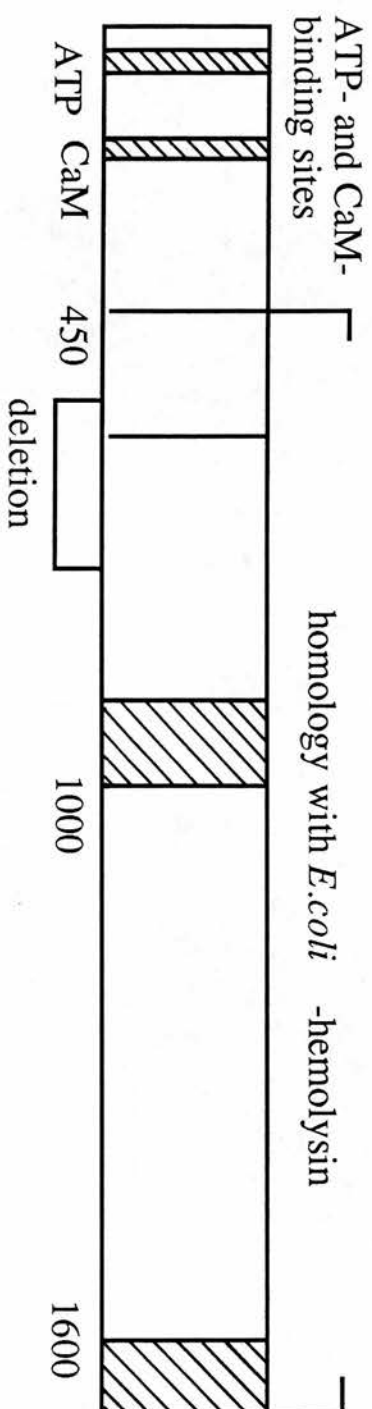
*Bordetella pertussis* adenylate cyclase is extracytoplasmic and several strains of the bacteria release considerable amounts of this protein into the culture medium (Hewlett et al., 1976). The enzyme is activated by eukaryote calmodulin and leads to the uncontrolled production of cAMP (Confer and Eaton, 1982). Many of the symptoms of whooping cough infection are due to an impaired immune response by the host. These symptoms are the lack of fever, an inadequate neutrophilic response and high incidence of bacterial secondary infections. Adenylate cyclase is responsible for these disease characteristics as the high levels of cAMP produced impair the ability of polymorphonuclear leukocytes and macrophages for chemotaxis, phagocytosis, superoxide generation and microbial killing. Adenylate cyclase has been shown to be essential in virulence as an insertion mutant deficient in AC was avirulent in an animal model of pertussis infection (Weiss et al., 1983) and introduction of a cloned AC gene into this mutant restored virulence (Brownlie et al., 1988).

The adenylate cyclase gene has been cloned and sequenced (Glaser et al., 1988) and it has the potential to encode for a protein of 200kDA.

The gene is shown in Figure 1.

**FIGURE 1.1**

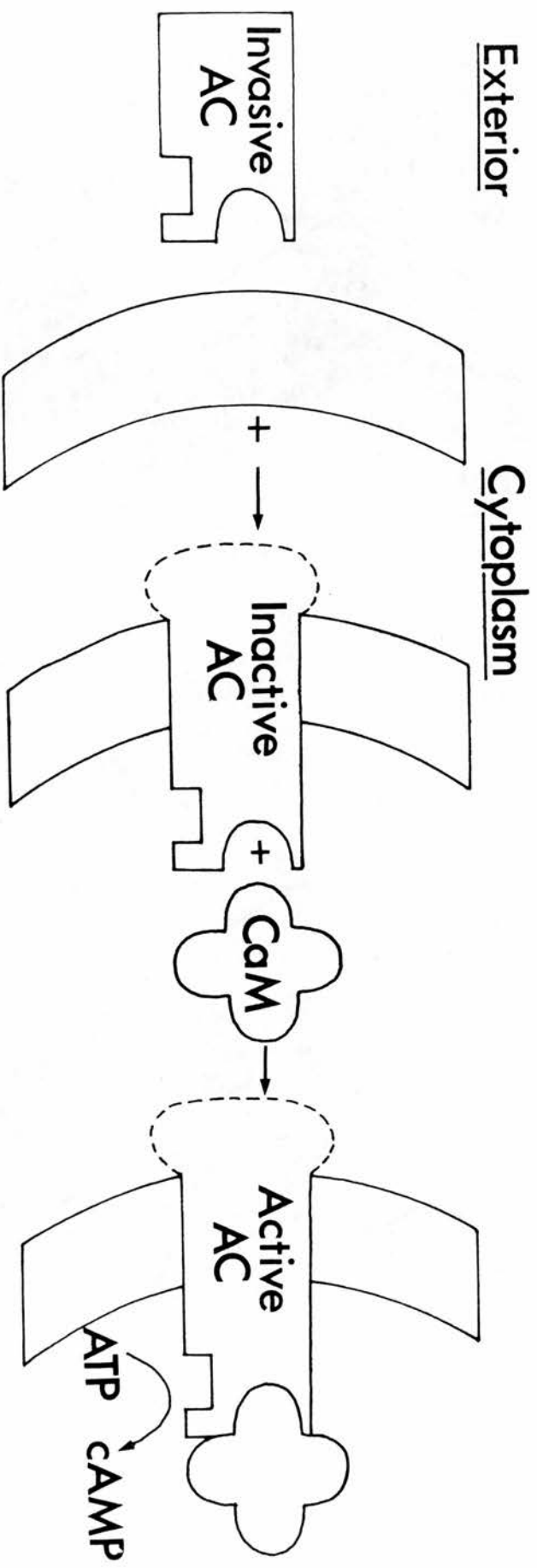
***B. PERTUSSIS* ADENYLATE CYCLASE GENE  
ORGANISATION**



**FIGURE 1.2**

**MECHANISM OF ACTION OF *B. PERTUSSIS*  
INVASIVE ADENYLATE CYCLASE**

**(ADAPTED FROM HANSKI, 1989)**



The 200kDa form of the protein is required for penetration (Hewlett et al., 1988) suggesting that the observed proteolytic cleavage of the 200kDa active form to a smaller 45kDa form which is non-toxic is irrelevant for both the secretion and production of a toxic form of the enzyme.

The protein penetrates the host cell directly from the cytoplasm and produces a transient increase in host-cell cAMP levels, the mechanism of cAMP production being shown in Fig. 1.2. This is contrasted by the toxins which act through ADP-ribosylation, which is a long-lasting effect. This suggests that the initial rise in host cell cAMP produced by AC is amplified by the increased production of cAMP by the latent ADP-ribosylation.

The adenylate cyclase can also act as a haemolysin. The 1250 C-terminal amino acids have a significant degree of homology with the *E. coli*  $\alpha$ -hemolysin and the hemolysin of *pasteurella hemolytica* (Glasser et al., 1988) with the homology being particularly pronounced between residues 640 and 910. However the relationship between the toxic and haemolytic activities of the enzyme remains to be clarified.

#### 1.2.4. Lipopolysaccharide endotoxin

There are two lipopolysaccharides produced by *B. pertussis*, LPS I and LPS II, each containing low molecular weight polysaccharides. LPS I contains a non-phosphorylated 3-deoxy-2,octulosonic acid (KDO) and LPS II contains phosphorylated KDO. Bacterial lipopolysaccharide endotoxins have the general structure shown in Fig. 1.3.

Milner et al., (1963) showed that *B. pertussis* LPS are long fibres of 7-8nm in diameter and are found either as loops or rings. By SDS polyacrylamide gel electrophoresis a mass of 5,000 has been calculated for this endotoxin (Peppler, 1984). LPS does not appear to play an important role in the pathogenesis of the disease; however, it is thought that some of the unpleasant, though mild, side-effects associated with pertussis whole-cell vaccination may be a result of this endotoxin.

**FIGURE 1.3**

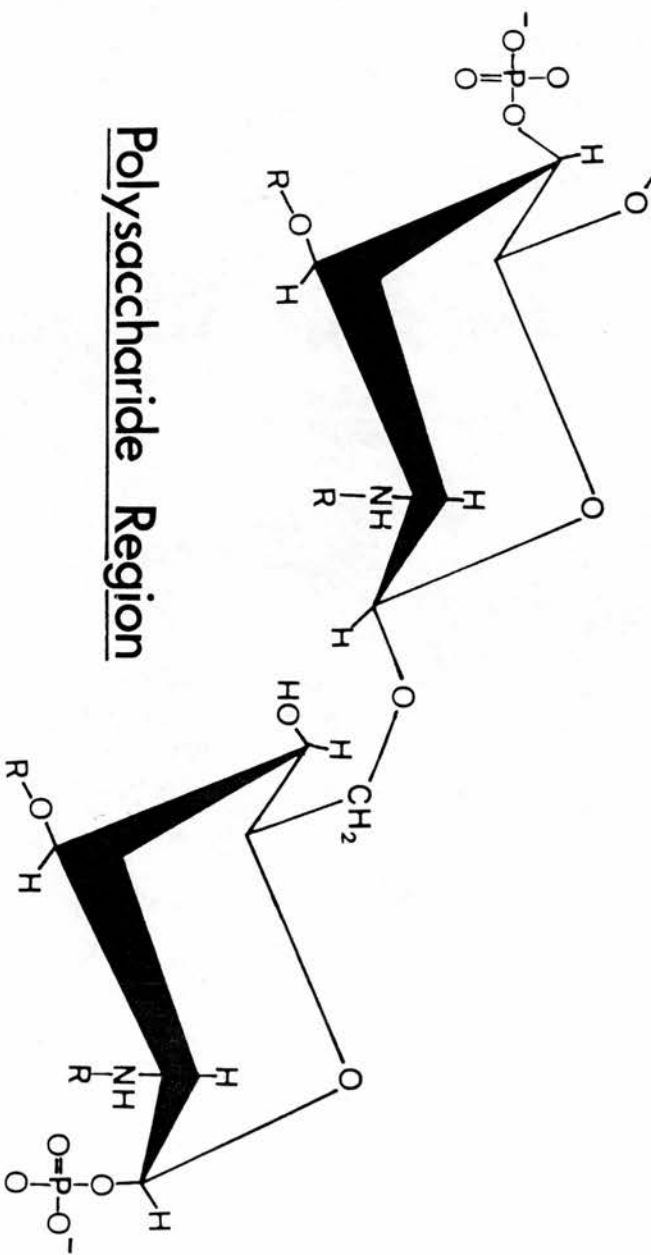
**GENERAL STRUCTURE OF BACTERIAL  
LIPOPOLYSACCHARIDE ENDOTOXINS**



REPEATING UNITS

-----KDO

## O-Specific Chain



## Polysaccharide Region

### 1.2.5. Dermonecrotizing (heat-labile) toxin

Heat-labile toxin (HLT) is a 56°C-labile protein. The protein is also known as dermonecrotizing toxin as it causes skin lesions. First discovered in 1909 by Bordet and Gengou, it is thought to be responsible for the changes in the respiratory tract mucosa typical of *B. pertussis* infection. HLT has an overall molecular mass of 102 000 and is composed of four subunits - two of 20,000 and the other two of 30,000 (Endoh et al., 1986).

The role of HLT in the pathogenesis of pertussis is again unclear, but recent evidence shows that the toxin's vasoconstrictive effects play a significant part in the initial stages of the disease. This HLT-induced vasoconstriction could result in both respiratory and peripheral circulatory failure leading to the decubediosis and petichial haemorrhage seen in HLT inoculated guinea-pig skin (Parton, 1986).

### 1.2.6. Trachael cytotoxin

Trachael cytotoxin is the only toxin produced by *B. pertussis* which has been shown to play a direct role in damaging the respiratory tract. Purified TCT can be shown to produce the ciliostasis and the specific ciliated cell-damage found in *B. pertussis* infection (Goldman et al., 1982).

This toxin is a small glycopeptide probably derived from the peptidoglycans of the cell envelope. Its estimated molecular weight is between 1,235 and 1,400. TCT appears to be released from *B. pertussis* by hydrolysis of peptidoglycan fragments from the peptidoglycan matrix of the bacteria itself and not by release from peptidoglycan precursors.

A summary of the biological active components of *B. pertussis* is shown in Table 1.2

TABLE 1.2                      BIOLOGICALLY ACTIVE SUBSTANCES PRODUCED BY *BORDETELLA PERTUSSIS*

<u>Protein</u>	<u>Molecular mass</u>	<u>Proposed role in the pathogenesis of whooping cough</u>
Agglutinogens	10 000 - 23 000	Role unclear but thought to be involved in adhesion of <i>B. pertussis</i> to host cells.
Filamentous haemagglutinin	220 000	Involved in the adhesion of <i>B. pertussis</i> to host cells.
Adenylate cyclase	200 000	Impairs host-cell defence mechanisms during <i>B. pertussis</i> infection.
Dermonecrotising Toxin	102 000	Vasoconstrictive effects. Thought to be important at the initial stages of the toxin disease.
Haemolysin	Unknown	Cell lysis and release of nutrients.
Lipopolysaccharide endotoxin	5 000	No suggested role in the pathogenesis of disease.
Tracheal cytotoxin	1235-1400	Ciliostasis and ciliated cell damage.
Pertussis toxin	117 000	Most important toxin involved in disease symptoms. Recent evidence suggests that most of the systemic disease symptoms are as a result of pertussis toxin.

### 1.3. The Structure and Function of Pertussis Toxin

#### 1.3.1. Introduction

In 1979, Pittman suggested that all of the harmful effects of *B. pertussis* infection are as a consequence of one specific antigen. Other diseases, such as tetanus and diphtheria, have this characteristic where tetanus toxin and diphtheria toxin respectively are responsible for the harmful effects of tetanus and diphtheria. If this is the case for pertussis then it has been suggested (Pittman, 1979) that pertussis toxin could be this antigen. As is shown in Table 1.4 pertussis toxin is involved in each stage of the disease after exposure of the host to the bacteria. Pertussis results as a consequence of the bacteria adhering to certain cell types, a process which involves a number of virulence factors, followed by the secretion of a number of toxins. It seems unlikely that pertussis is caused by a single exotoxin as *B. bronchiseptica* causes a pertussis-like disease in animals (kennel cough) and *B. parapertussis* produces a mild form of whooping cough and neither produce pertussis toxin or a pertussis toxin-like protein. Pertussis toxin cannot be administered to children but if this was done the patient would probably not get whooping cough, adding to the evidence against pertussis being a disease mainly caused by one toxin.

Pertussis toxin exhibits a wide number of biological activities, the most important of which is the activation of mammalian adenylate cyclase. The manifestations of the effects of pertussis toxin in many animals include promotion of lymphocytosis and leukocytosis, haemagglutination, enhances susceptibility to histamine, serotonin, endotoxin and cold, enhancing insulin secretion from pancreatic cells, inducing hypoglycaemia, inhibiting adrenalin induced hyperglycaemia and associated adjuvancy effects, especially for IgE. (For review see Wardlaw and Parton, 1983). However, it was demonstrated by Irons and MacLennan (1979) that purified pertussis toxin did not retain all of the biological activities shown above - only some of them, suggesting that the others may be as a result of contamination with other proteins.

As a consequence of these diverse biological activities the toxin was given a wide number of names in the past, each reflecting an individual activity. These include

TABLE 1.3    HOST-PARASITE RELATIONSHIP IN PERTUSSIS (From Wardlaw and Parton, 1988)

Host		Parasite		
Clinical Stage	Outcome of disease if halted at this stage	Event	Component Involved	Activity
Exposure	Nil	Transmission	Whole cell	
Incubation	Asymptomatic Infection	Attachment and growth	FHA PT AGGs(?)	Attachment and nutrient acquisition
Catarrhal	Atypical pertussis	Secretion of toxins	OMPs(?)	
Paroxysmal	Classical pertussis		TCT HLT ACT PT FHA PT	Disruption of host defences and initiation of local and systemic pathology
	Convalescence	Encounter with host immune response		
Severe pertussis	Bronchopneumonia	Bacterial expulsion or death		
	Secondary Infections (usually fatal)			

AGGs 2 and 3

AGGs 2 and 3

lymphocytosis promoting factor, islet-activating protein, histamine sensitisation factor, pertussigen and finally pertussis toxin. Pertussis toxin is now the name universally accepted, which may not be completely appropriate in view of the other toxins of the bacteria.

### 1.3.2. The structure of pertussis toxin

In recent years the reported molecular masses of pertussis toxin have been variable, with values ranging from 171 kDa (Cowell *et al.*, 1982) down to 100kDa (Morse and Morse, 1976). The molecular mass of 117kDa calculated by Tamura *et al.*, (1982) is the only precise result. The method used was analytical ultracentrifugation. They found that pertussis toxin was homogeneous in 2M urea at acid pH, had a sedimentation coefficient of 6.6S and molecular mass of 117 kDa as measured by sedimentation equilibrium at two vector speeds. A summary of the molecular mass values are shown in Table 1.4.

The toxin is a very stable protein, the holotoxin being resistant to proteases (Peppler *et al.*, 1985) although susceptibility to the action of protease increases as the holotoxin dissociates into subunits. The toxin retains its biological activity in solutions containing 2M urea, but as the concentration increases, biological activity is lost as the toxin dissociates (Tamura *et al.*, 1982).

The toxin has been shown by SDS polyacrylamide gel electrophoresis (SDS PAGE) to be composed of five different subunits termed S-1 to S-5 on the basis of their decreasing molecular mass. Tamura *et al.*, (1982) showed via densitometric analysis of Coomassie blue stained pertussis toxin subunits that the molar ratio of the subunits S-1, S-2, S-3, S-4 and S-5 within the holotoxin was 1:1:1:2:1. They calibrated for each subunit the relationship between  $\mu\text{g}$  of subunit and Coomassie blue staining. This molar ratio was also demonstrated by Sekura *et al.*, (1983) by a similar procedure. Rappuoli and co-workers (1987) further confirmed this subunit stoichiometry, their evidence being based on the association of the toxin subunits in different urea concentrations. Although this stoichiometry has been universally accepted, it is based upon rather weak evidence. Scanning of Coomassie

TABLE 1.4      COMPARISON OF MOLECULAR MASSES CALCULATED FOR PERTUSSIS TOXIN AND SUBUNITS

Apparent Molecular Weight $\times 10^3$ Subunit						Total Molecular Weight	Reference
S1	S2	S3	S4	S5	S5/4*		
23.5	19.3	17.4	-		13.4	100	Morse & Morse (1976)
27.2	22.4	21.1	-		12.6	109	Irons & MacLennan (1979)
28	23	22	11.7	9.3		106	Tamura et al (1982)
34	30	29	-		26	171	Cowell et al (1982)
30	26.1	25.4	-		13.9	123	Sekura et al (1983)
26.1	25.3	22	-		13.2	113	Sekura et al (1983)
28	24.5	23.5	-		14.0	118	Perera et al (1985)
28.2	24.8	24.3	12.2	13.9		116	Pepler et al (1985)
26.3	24.4	22.7	12.2	11.3		109	Pepler et al (1985)
26.0	21.9	21.9	12.1	11.0		105	Locht & Keith (1986)
26.2	21.9	21.9	12.1	10.9		105	Nicosia et al (1986)

Taken from Irons and Goringe (1988)

stained gels is based on the assumption that all proteins have the same capacity for dye binding, and the association of subunits in dissociating agents does not directly demonstrate the stoichiometry of the protein. This stoichiometry might be confirmed by ultracentrifugation of the holotoxin and isolated subunits. Chemical cross-linking reagents would also be invaluable in confirming this proposed stoichiometry.

The overall subunit arrangement of pertussis toxin is similar to that of many other bacterial exotoxins in that it has the typical A-B type structure (Gill, 1976; van Heyningen, 1982; Burns and Manclark, 1985; Burns, 1988). The A-protomer is enzymically active and is composed of S-1 alone. The binding, B-oligomer is involved in receptor binding and internalisation of the S-1 subunit. This part of the toxin is made up of the remaining subunits. The proposed stoichiometry of toxin subunits is shown in Fig. 1.4. If the holotoxin is exposed to 2M urea S-1 will dissociate from the B-oligomer. Further exposure of the B-oligomer to 5M urea causes the binding component to dissociate into two dimers of S-2 and S-4 (D1) and of S-3 and S-4 (D2) and a free S-5. Finally exposure to 8M urea causes the two dimers to dissociate into their constituent subunits (Rappuoli and Silvestri, 1987).

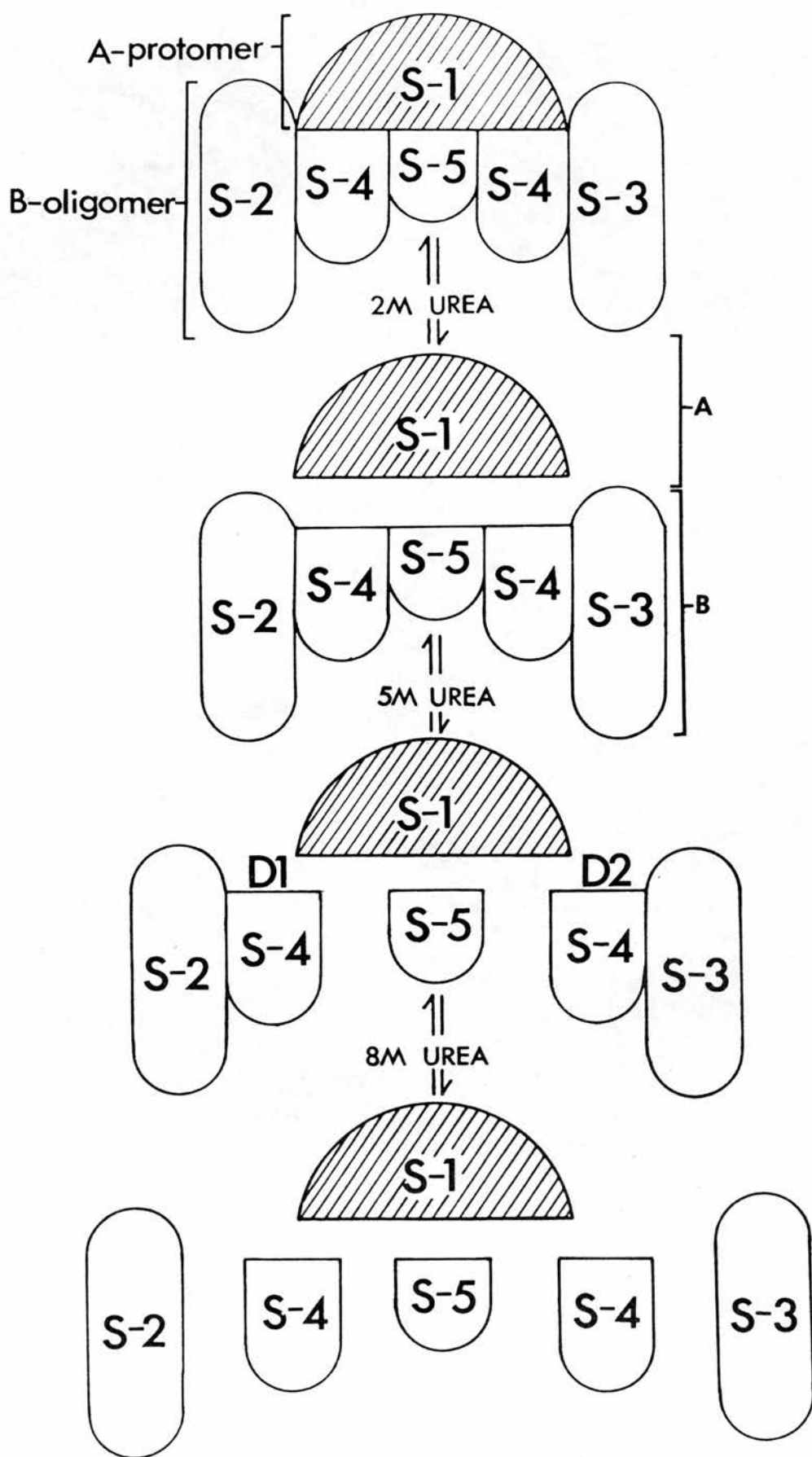
Montecucco *et al.*, (1986), using the technique of hydrophobic photolabelling of pertussis toxin subunits, suggested a model for the spatial organisation of the subunits. This technique involves the use of radioactive, photoreactive, phospholipid analogues which were interdispersed among detergent micelles. These phospholipid analogues can cross-link to the protein thereby showing the toxin's detergent-binding sites. This approach is very sensitive and can be used to map the hydrophobic regions on the protein which are involved in interaction with lipids. They showed that subunits S-2, S-3, S-4 and S-5 probably all interacted with lipid, suggesting that each of these subunits is involved in membrane insertion of the toxin. Subunits S-2 and S-3 were shown to penetrate the hydrophobic core of the micelle and subunit S-4 was found at the polar head-group region of the phospholipids. S-1 was not labelled in this model suggesting that the B-oligomer may shield S-1 from interacting with lipids. The model proposed is shown in Fig. 1.5.

This proposed pentameric binding domain, the B-oligomer, shows many similarities with the proposed structure of cholera toxin where five identical B-



**FIGURE 1.4**

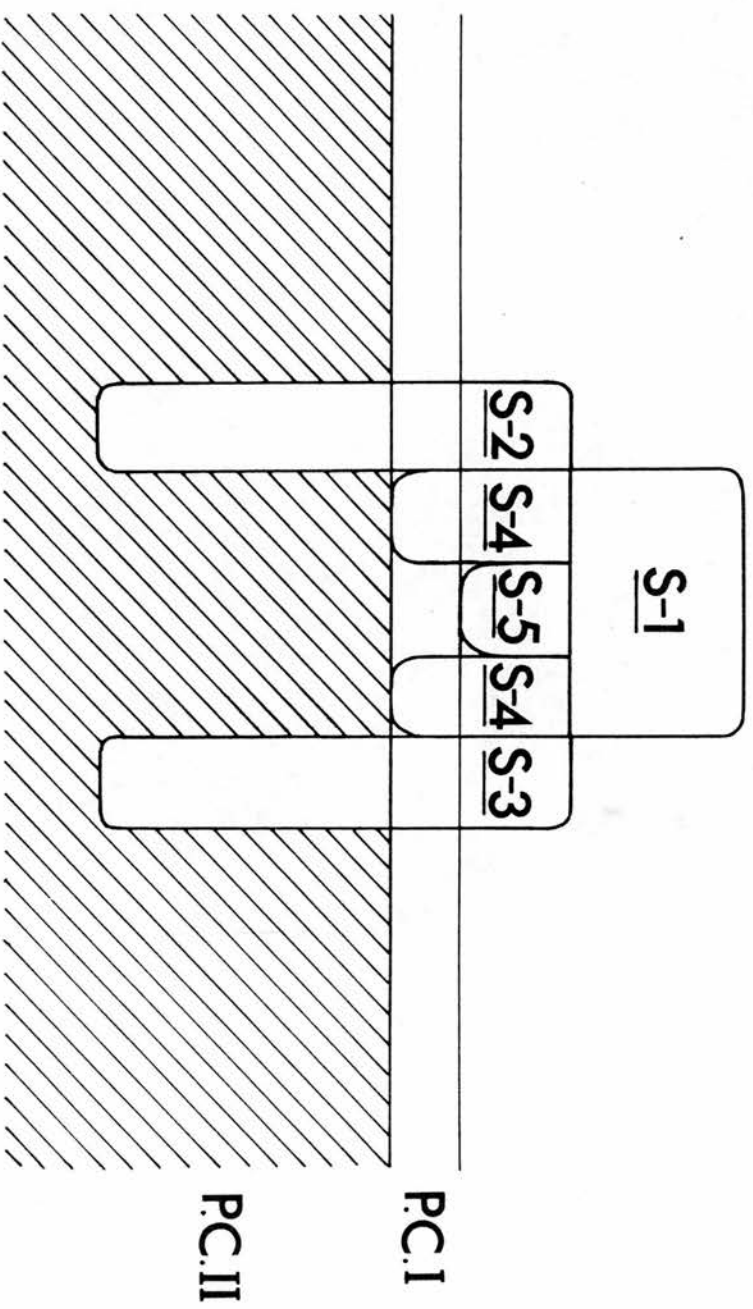
**PROPOSED SUBUNIT STOICHIOMETRY OF  
PERTUSSIS TOXIN, ADAPTED FROM  
RAPPUOLI AND SILVESTRI (1987)**



**FIGURE 1.5**

**INTERACTION OF THE B-OLIGOMER SUBUNITS WITH THE  
HOST-CELL PLASMA-MEMBRANE.**

**ADAPTED FROM MONTECUCCO ET AL, (1986)**



subunits are arranged around the A-subunit, which sits above the plane of the binding component (van Heyningen, 1977). The S-1 subunit of pertussis toxin may occupy a similar position, but this has not yet been established. Work with reversible cross-linking reagents of different molecular spans should help to clarify this proposed structure. This work was undertaken in this project and the results obtained shown in Chapter 6.

### **1.3.3. Molecular cloning of pertussis toxin genes and its primary structure**

The complete amino acid sequence of all the toxin subunits have been derived from the nucleotide sequences (Locht and Keith, 1986; Nicosia *et al.*, 1986). The calculated molecular masses of the mature subunits are S-1 26 024 ; 21 924 for S-2; 21 873 for S-3; 12 058 for S-4 and 11 013 for S-5 (Locht and Keith, 1986). The S-1 subunit contains 234 amino acids, S-2 and S-3 both contain 199 amino acids, S-4 contains 110 amino acids and 100 amino acids for S-5. From the proposed stoichiometry discussed above, that is two molecules of S-4 per molecule of toxin, the overall calculated molecular mass is 104 950. This is in comparison with the value of 117 000 derived from analytical ultracentrifugation (Tamura *et al.*, 1982), this discrepancy of 12 000 may be due to the existence of another subunit within this model.

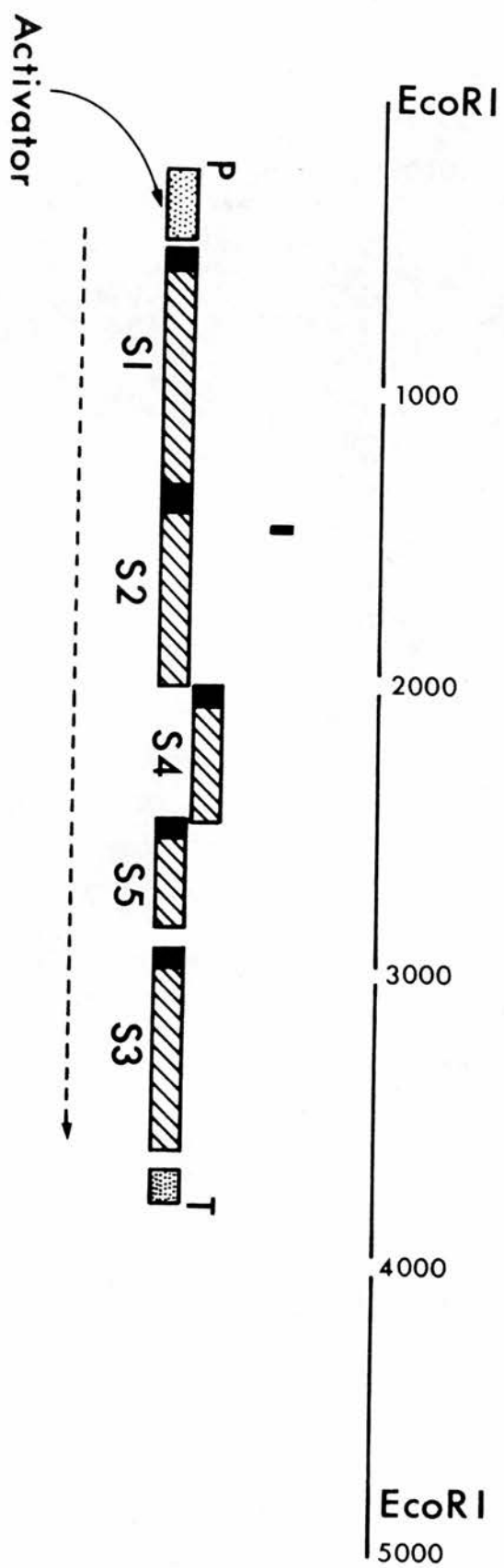
The genes encoding all of the subunits are located within a 3.2Kbase segment of the bacterial genome (Locht and Keith 1986; Nicosia *et al.*, 1986). The gene coding for S-4 overlaps that of S-2 and S-5 (see Fig. 1.6). The arrangement of the five genes are typical of a bacterial operon in that the genes are flanked by a promotor region and a termination signal is found downstream from the five genes (Rappuoli and Silvestri, 1987). Upstream of the promotor is a piece of DNA which is the target for a trans-activating protein (vir). This protein is responsible for switching on and off transcription of the pertussis toxin subunits and other virulence genes.

Each of the five subunits are synthesised as precursor proteins with a preceding signal peptide, as would be expected for a secreted protein. This suggests that each of

**FIGURE 1.6**

**ORGANISATION OF THE PERTUSSIS TOXIN OPERON**

**(LOCHT AND KEITH, 1986; NICOSIA ET AL, 1986)**



the subunits  $\omega$  synthesised individually, whereupon they are secreted into the periplasm where their signal peptides are cleaved and the mature subunits assembled into the holotoxin. Once assembled in the periplasm, the toxin is released into the extracellular medium (Nicosia et al., 1986; Nicosia and Rappuoli, 1987).

There is a very high degree of homology between subunits S-2 and S-3. The two subunits show 70% amino acid homology and 75% homology at the DNA level. This homology suggests that the two genes were generated from a gene duplication of one ancestral gene, which have subsequently undergone mutations leading to the production of two individual subunits. This high degree of similarity is reflected in both the secondary structure of the two subunits and also in their hydrophilicity<sup>123</sup> however, as shown by Tamura et al., (1989) one subunit cannot substitute for the other in the enzymically active toxin.

The amino acid sequence of the S-1 subunit has been compared to the active subunits of many other bacterial exotoxins. Two regions of significant homology can be identified between pertussis toxin and both cholera and *Escherichia. coli* heat-labile toxins. These regions of homology are seen at analogous regions within all three toxins.

### **Region 1**

Pertussis	S-1 subunit	8	Tyr	Arg	Tyr	Asp	Ser	Arg	Pro	Pro	15
Cholera	A-subunit	6	Tyr	Arg	Ala	Asp	Ser	Arg	Pro	Pro	
E. coli LT	A-subunit	6	Tyr	Arg	Ala	Asp	Ser	Arg	Pro	Pro	

### **Region 2**

Pertussis	S-1 subunit	51	Val	Ser	Thr	Ser	Ser	Ser	Arg	Arg	58
Cholera	A-subunit	60	Val	Ser	Thr	Ser	Leu	Ser	Leu	Arg	67
E. coli LT	A-subunit	60	Val	Ser	Thr	Ser	Leu	Ser	Leu	Arg	67

These similarities at the amino acid level are not reflected in the primary sequence as pertussis toxin uses different triplet codons to that of cholera toxin and LT toxin. No other sequence homologies could be found between pertussis toxin S-1 subunit and the other two A-subunits. The DNA sequences of the other pertussis



toxin subunits show no resemblance to any of the other ADP-ribosylating toxins.

#### 1.3.4. The role of each subunit

The B-oligomer of pertussis toxin is responsible for binding of the toxin to a specific receptor on the host cell surface and also facilitates the entry of the S-1 subunit into the cell. The B-oligomer has been shown to bind to a 165kDa glycoprotein on Chinese hamster ovary cells (CHO cells) (Brennan *et al.*, 1988; Burns, 1988). The glycoprotein has been termed an N-acetylneuraminic acid Galactose  $\beta$ -4 N-acetylglucosamine (Neu Ac Gal  $\beta$  Glc N Ac) oligosaccharide sequence suggesting that this carbohydrate may be important in the binding of toxin to host cells. The S-1 subunit does not interact with the cell membrane (Montecucco *et al.*, 1986) and the mechanism whereby it enters the cell is unknown (see below). Once within the cell the S-1 subunit activates adenylate cyclase by ADP-ribosylation of Gi, a regulatory protein of the adenylate cyclase complex. The details of this mechanism will be further discussed in a later section.

#### 1.3.5. Interaction of pertussis toxin with the cell membrane

The mechanism of B-oligomer binding to a receptor and entry of the S-1 subunit has not been demonstrated. The lymphocytosis activity of the toxin is thought to be the result of the interaction of the B-oligomer with host-cell receptors (Nogimori *et al.*, 1984a and b) but these receptors have not yet been identified.

The toxin has been shown to bind to the oligosaccharide regions of several glycoproteins such as haptoglobin and fetuin (Irons and MacLennan, 1979; Sekura and Zhang, 1985). Armstrong *et al.*, (1987) further investigated the interaction of toxin with fetuin in order to characterise its binding to carbohydrate and to demonstrate its lectin-like properties. They concluded that two sugars - Neu Ac and Glc NAc were the most important residues in toxin binding to complex carbohydrates. It was suggested that the toxin's interaction with fetuin, and possible specific membrane

receptors, also depends upon the correct orientation of the sugar groups and not upon electrostatic interactions with the negatively charged carbohydrate groups.

As described above the toxin binds to a 165kDa glycoprotein with a terminal Neu Ac -----> Gal $\beta$ 4GlcNAc on CHO cells (Brennan *et al.*, 1988). This further demonstrates that the toxin preferentially interacts with N-linked oligosaccharides (Irons and MacLennan, 1979; Sekura and Zhang, 1985).

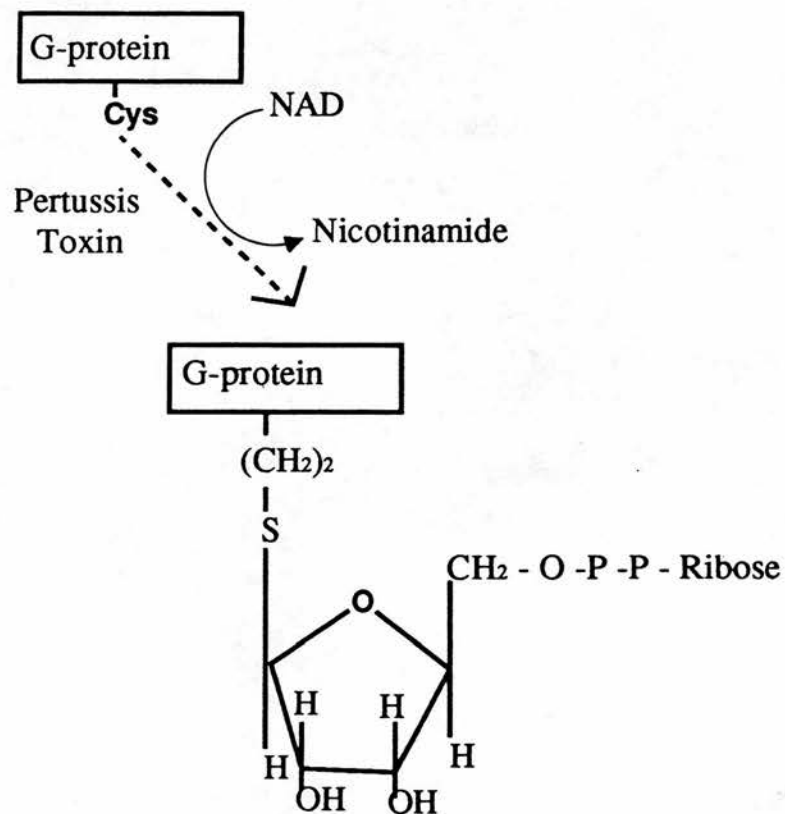
The oligosaccharide binding site on the B-oligomer has been further narrowed down to the S-3/S-4 dimer as this is the region shown to directly interact with the 165kDa protein. Both of the dimers, however, are capable of haemagglutinating erythrocytes and can bind to fetuin. This suggests that there are multiple lectin-like binding sites, with different specificities, on the B-oligomer.

This work indicates that glycoproteins may serve as receptors for pertussis toxin on other cell types. Brennan *et al.*, (1988) have suggested that as pertussis toxin plays a role in the adherence of *B. pertussis* to host cells, similar glycoproteins to those discussed above may serve as attachment sites for the bacteria.

### 1.3.6. The entry of toxin into the cell

It is essential for S-1 to cross the cell membrane to gain access to adenylate cyclase, which is located within the inner membrane. This penetration is likely to involve hydrophobic interactions between the toxin and the hydrophobic regions of membrane lipids (Montecucco *et al.*, 1986). As the S-1 subunit has been shown not to interact with membrane lipids, the B-subunits must shield the active peptide from membrane interaction.

Several possible mechanisms for active peptide entry have been suggested for cholera toxin; methods which may perhaps also apply for pertussis toxin. Firstly, van Heyningen and King (1975) suggested that there is no specific mechanism involved in the internalisation process but that, at a high local concentration of toxin, a few molecules of toxin must enter the cell by some random process. Gill (1976) suggested that the B-subunits could form a pore within the membrane through which the active subunit could enter the cell; however, Montecucco *et al.*, (1986) have shown, as



**FIGURE 1.7**

**MECHANISM OF PERTUSSIS TOXIN CATALYSED ADP-RIBOSYLATION**

described above, that the subunits of the B-oligomer do not all penetrate detergent micelles, demonstrating the impossibility of pore production. Another possible mechanism for the entry of S-1 is endocytosis, but there is no evidence for this as the toxin has never been found within endosomes. The model of greatest interest at present is that once the B-oligomer is bound to a specific membrane glycoprotein it will become internalised along with this glycoprotein in membrane regeneration. This would enable the toxin to cross the membrane and reach the cytosolic side. There is a noticeable lag period between the binding of the toxin to the cell membrane and expression of activity (Katada and Ui, 1980). This lag period could be attributed to the time taken for internalisation of the toxin. With cholera toxin the lag is also at least partly due to the time it takes for the active peptide to separate from the rest of the toxin. This may also be the case for pertussis toxin but the overall mechanism of its entry has still not been demonstrated.

#### **1.3.7. ADP-ribosylation and activation of adenylate cyclase**

In 1979 Katada and Ui demonstrated that pertussis toxin had a marked effect upon the receptor-mediated production of cAMP. This was later shown to result from the toxin-catalysed ADP-ribosylation of a 41kDa membrane-bound protein (Katada and Ui, 1982a). ADP-ribosylation involves the enzyme-catalysed breakdown of  $\text{NAD}^+$  to ADP-ribose and nicotinamide with the subsequent covalent modification of a membrane protein via the addition of ADP-ribose to a cysteine residue on this protein (West *et al.*, 1985).

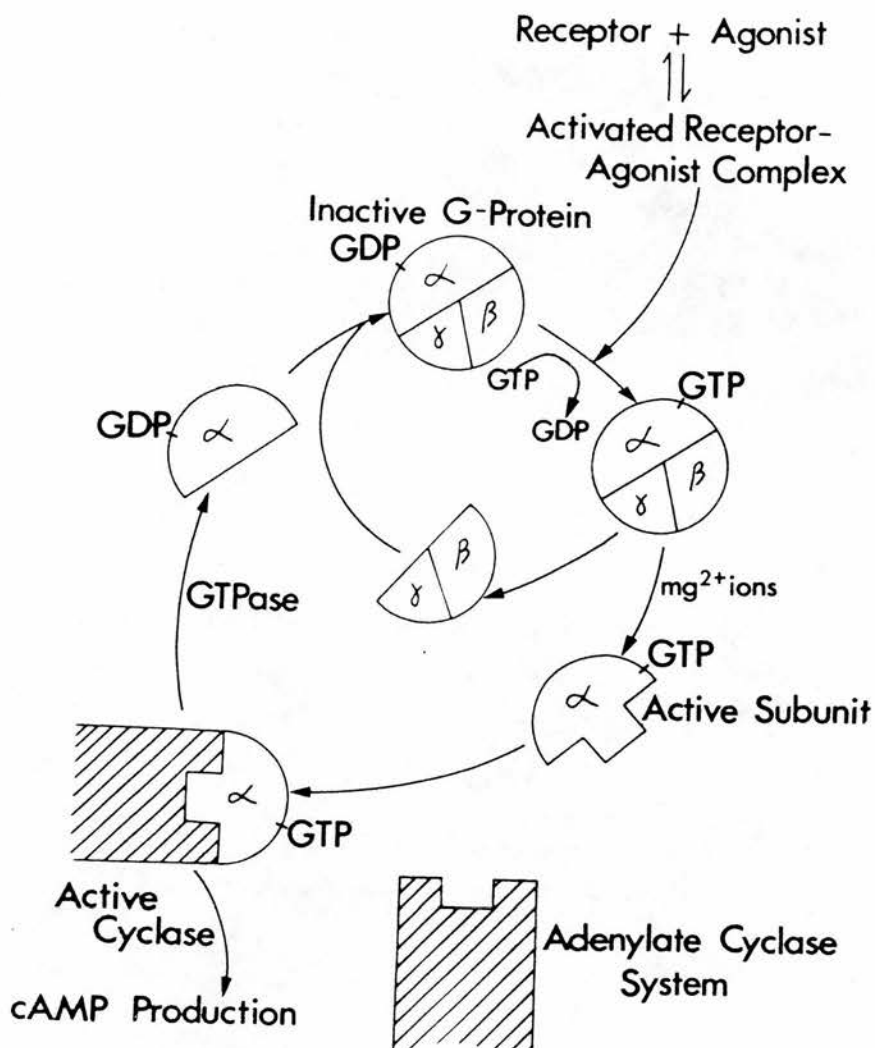
ADP-ribosylation is discussed further in Chapter 4. This pertussis toxin catalysed ADP-ribosylation is responsible for the reversal of receptor-mediated inhibition of adenylate cyclase activity leading to an increase in cAMP concentration within the cell. The mechanism for toxin catalysed ADP-ribosylation is shown in Fig. 1.7.

The main protein substrate for ADP-ribosylation is  $\text{Gi}$ . This protein is involved in regulation of adenylate cyclase and is normally under the control of inhibitory hormones. There are at least two G-proteins involved in the regulation of adenylate

cyclase, namely Gi - the inhibitory regulatory GTP-binding protein and Gs - the stimulatory regulatory GTP-binding protein, Gs being the substrate for cholera toxin. These G-proteins act as intermediates in the hormonal regulation of secondary messenger concentrations within the cell (Bokoch *et al.*, 1982; Neer and Clapham, 1988; Milligan, 1988). Rodbell and co-workers (1971) were the first group to demonstrate the requirement for guanine nucleotides in the activity of certain hormones in hepatocytes and following these initial observations many G-proteins have been isolated, which, although each is unique, are very similar in both structure and function.

Both Gi and Gs have three different subunits  $\alpha$ ,  $\beta$ , and  $\gamma$  (Hildebrandt, 1984), with an overall molecular mass of between 80-90kDa. The  $\alpha$ -subunit binds guanine nucleotides, Gi  $\alpha$  having a molecular mass of 41kDa. Katada and Ui, (1982) were the first to show that pertussis toxin catalysed the ADP-ribosylation of this protein within rat C6 glioma cells, when the cells are incubated with [ $\alpha$ - $^{32}$ P] NAD $^{+}$ . The toxin catalysed the transfer of radioactive ADP-ribose to this membrane protein.

The  $\alpha$ -subunit of Gs has a molecular mass in the range of 42-45kDa. The primary sequences of Gs $\alpha$  and Gi $\alpha$  are highly conserved and this conservation is carried through to the tertiary structure (Milligan, 1988). Manning and Gilman, (1983) suggested that there was a single  $\beta$ -subunit type, but this was later disproved by Sternweiss and Robinshaw (1984), who were the first group to show that there were distinct  $\beta$ -subunits for each  $\alpha$ -subunit. Molecular cloning techniques have subsequently confirmed the presence of at least two distinct  $\beta$ -subunit genes (Sugimoto *et al.*, 1985; Fong *et al.*, 1987). Very little is known about the  $\gamma$ -subunit. Immunological evidence (Hildebrandt *et al.*, 1985) suggests that there are a number of different forms, each being a small polypeptide of about 8kDa. In every situation so far studied, the relevant  $\gamma$ -subunit remains tightly bound to its  $\beta$ -subunit. The purpose



**FIGURE 1.8**

**ACTIVATION/DEACTIVATION PROCESS OF THE ADENYLATE CYCLASE CATALYTIC UNIT.**

**ADAPTED FROM MILLIGAN (1988)**

of this  $\beta\gamma$ -complex is probably to anchor the associated  $\alpha\beta\gamma$ -complex in the cell membrane and may also be involved in communication with the rest of the cyclase complex (Sternweiss, 1986).

The activation of Gi or Gs is dependant upon both guanine nucleotides and  $Mg^{2+}$  ions (Hildebrandt and Birnbaumer, 1983). This activation is brought about by the dissociation of the  $\alpha$ -subunit from the  $\alpha\beta\gamma$ -complex. This activation is shown in Figure 1.8.

The  $\alpha\beta\gamma$  form of Gi is known as the resting state of this protein complex (Milligan, 1988). The  $\alpha$ -subunit becomes active when it is dissociated from the  $\beta\gamma$ -complex (Hildebrandt *et al.*, 1983; Khan and Gilman, 1984) and bound GDP is replaced with GTP. The release of GDP is the rate-limiting step in the activation process (Gilman, 1987; Milligan, 1988). Once GTP is bound to the  $\alpha$ -subunit, and in the presence of  $Mg^{2+}$  ions, the  $\alpha$ -subunit can then interact with adenylate cyclase and alter the rate of synthesis of cAMP. There is a cyclical pattern of activation and deactivation which is completed when bound GTP is hydrolysed to GDP by the intrinsic GTPase activity of the  $\alpha$ -subunit. This  $\alpha$ -subunit-GDP form can then reassociate with the  $\beta\gamma$ -complex reverting Gi back to its resting state. The effect of both pertussis and cholera toxins is to inhibit the GTPase of Gi and Gs respectively maintaining the active conformation of both proteins (Cassel and Selinger, 1977) and resulting in an unregulated increase in the production of cAMP.

### **1.3.8. Other protein substrates for pertussis toxin**

Pertussis toxin catalyses the ADP-ribosylation of many other G-proteins. As with Gi, these G-proteins also serve as transducers communicating between receptors and effectors in mammalian cells. Again, as with the adenylate cyclase system, the first step in this transduction process is the recognition of a specific receptor by some



extracellular signal such as hormones, neurotransmitters and autocoids. The receptor can then interact with the effectors of the system. These are generally other enzymes controlling the production of secondary messenger substances such as cAMP, diacylglycerol or inositol phosphates, or secondly controlling ion channels which regulate ion concentration within the cell.

These cascade processes eventually lead to other cellular activities such as muscle relaxation and contraction, dermataxis, exocytosis and endocytosis, energy metabolism, cell proliferation and cell differentiation (Ui, 1988). As pertussis toxin prevents G-protein inhibition of several of these cascade processes it can have far-reaching effects upon the physiological regulation of cell function.

After Gi, the next protein substrate found for pertussis toxin was transducin. Transducin is also a GTP-binding regulatory protein. It is found in the retina and regulates the activity of a cyclic GMP phosphodiesterase via light activated rhodopsin. This protein is another  $\alpha\beta\gamma$  heterotrimer and is structurally homologous to the adenylate cyclase GTP-binding regulatory proteins. Transducin is also ADP-ribosylated by cholera toxin but the sites of ADP-ribosylation by each toxin are distinct (Manning *et al*, 1984). The visual signaling converting the GTP-bound  $\alpha$ -subunit plus  $\beta\gamma$ -dimer form to the resting state is prevented by the pertussis toxin catalysed covalent modification of the  $\alpha$ -subunit. The  $\alpha$ -subunit of transducin has a molecular mass of 39kDa.

Katada and Ui (1983), further identified another two pertussis toxin GTP-binding protein substrates from rat brain. These proteins are again  $\alpha\beta\gamma$  heterotrimers and are known as Go and  $G_{\alpha_{40}\beta\gamma}$ . The roles of either of these two proteins in the central nervous system is unknown.

The phospholipase C enzyme is another protein under the control of GTP-binding proteins (Ui *et al*, 1985; Rappuoli and Silvestri, 1987). These G-proteins are known as Gp and have been identified as substrates for pertussis toxin catalysed ADP-ribosylation (Muryama and Ui, 1985; Kurose and Ui, 1985). Phospholipase C is



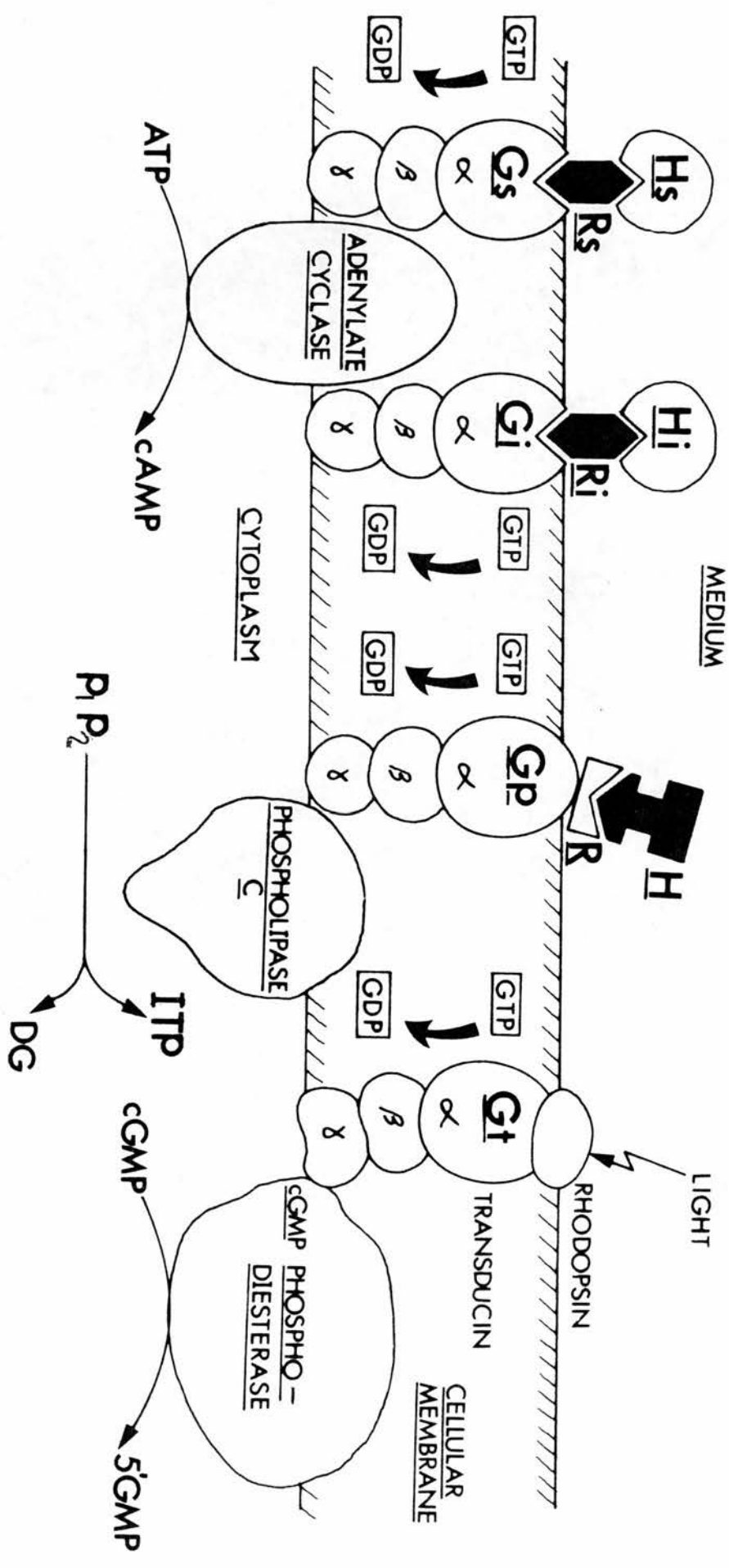
responsible for the production of two secondary messengers - inositol triphosphate and diacylglycerol, within the cell. A summary of pertussis toxin catalysed ADP-ribosylation is shown in Fig. 1.9.

Many of the clinical effects of *B. pertussis* infection can be attributed to ADP-ribosylation of G-proteins. For example, when animals which have been pretreated with pertussis toxin are challenged with histamine, the normal compensatory physiological response does not occur (Ui, 1988). The pertussis toxin catalysed ADP-ribosylation prevents the  $\alpha$ -adrenergic (Ri) induced release of catecholamine and the histamine induced vasodilation is eventually fatal. This effect is known as histamine sensitisation, pertussis toxin also being known as histamine-sensitisation factor (Okajuma and Ui, 1984; Okajuma et al., 1985). The  $\alpha$ -adrenergic blockage may also be responsible for the toxin induced hypoglycaemia (Yajima et al., 1981). As the  $\alpha$ -adrenergic system is responsible for inhibition of insulin secretion, once the system becomes non-functional unregulated insulin secretion can result. This may explain the islet-activating activity of the toxin (Katada and Ui, 1979), this activity again probably resulting from the pertussis toxin catalysed ADP-ribosylation of Gi. Pertussis toxin blockage of the  $\alpha$ -adrenergic response therefore results in adrenal increasing insulin production by a  $\beta$ -adrenergic (Rs) mechanism.

Pertussis toxin can modify the growth of CHO cells causing the cells to cluster and overlap in a similar way to transformed cells. This effect on CHO cells has been related to ADP-ribosylation (Guerrant et al., 1974). The transformation process may be interesting as the p21 ras oncogene products show similarities to G-proteins. The p21 proteins are a group of highly related proteins which show amino acid homologies with both transducin and the GTP-binding elongation factors (Lochrie et al., 1985). The p21 proteins are membrane bound and GTP-binding, but they have unknown functions. It has been suggested that they may have similar functions to Gi and Gs. ADP-ribosylation of the ras proteins could therefore result in some cellular enzymes being permanently active leading to the uncontrolled cell-proliferation associated with transformed cells. ADP-ribosylation of the p21 ras proteins has not been detected by

**FIGURE 1.9**

**SUMMARY OF PERTUSSIS TOXIN CATALYSED  
ADP-RIBOSYLATION.  
ADAPTED FROM RAPPUOLI AND NICOSIA (1987)**



either cholera (Gill and Wodkalis, 1985) or pertussis toxin (Ui, 1988). The yeast *Saccharomyces cerevisiae* produces a ras protein which has a molecular mass greater than p21 and has been shown to be a regulator of adenylate cyclase. However, again there is no evidence that this protein is ADP-ribosylated by pertussis toxin or any of the other bacterial toxins.

Pertussis toxin can therefore be used as an effective biological tool to help in the understanding of cellular communication with the toxin being used for the identification of regulatory G-proteins.

Most of the effects of pertussis toxin, as discussed above, are due to ADP-ribosylation but it is important to note that some of the activities such as lymphocytosis and leukocytosis and adjuvancy effects for IgE are thought to depend only on binding of toxin (Nogimori *et al.*, 1984a and b).

#### 1.4. Other toxins

Many of the other bacterial protein toxins show similarities to pertussis toxin in three main ways. Firstly, in their two-component A-B structures, secondly, in their mechanisms of action and finally in the way they bind to cells. Examples of other toxins which exert their biological activities by ADP-ribosylation are cholera toxin, *E. coli* heat labile toxin, diphtheria toxin and exotoxin A of *Pseudomonas aeruginosa*. Vaccines to the diseases associated with these toxins are made up of toxoids of these ADP-ribosylating toxins, for example, cholera and diphtheria, and these vaccines have been found to be practically, if not totally effective. Each of these toxins will be discussed in the following section along with the neurotoxins of tetanus and botulinum. A table showing the similarities of these toxins is shown in Table 1.4.

##### 1.4.1. Cholera toxin

Infection of the human gastrointestinal tract by the bacterium *Vibrio cholera* results in the disease cholera. The symptoms of this disease are severe diarrhoea resulting in extensive loss of fluid, which may be as much as twenty litres per day,

causing severe dehydration and in some cases causing death only a few hours after the onset of the disease symptoms. *V. cholera*, like *B. pertussis*, is a non-invasive organism. The disease symptoms are attributed to cholera toxin, a potent exotoxin produced by the bacteria. The way in which cholera toxin exerts its biological effects is very complex but they all result from activation of the adenylate cyclase of intestinal cells. This activation results in elevation of cellular cAMP levels and disruption of the normal ion fluxes of the intestinal epithelium (Middlebrook and Dorland, 1984).

Cholera toxin activates adenylate cyclase via Gs, the stimulatory regulatory protein of this complex. The toxin catalyses the ADP-ribosylation of this GTP-binding protein maintaining it in its  $\alpha$ s-GTP bound active conformation, resulting in the accumulation of cAMP within cells (Pfeuffer, 1977). The amino acid which is modified by cholera toxin is arginine (Moss and Vaughn, 1977).

The toxin is composed of one A-subunit and five identical B-subunits. Each of the B-subunits has a molecular mass of 11,600 and the A-subunit 27,000. The A-subunit undergoes proteolytic cleavage upon activation producing an A1 peptide of 22,000 and an A2 peptide of 5,000 molecular mass (van Heyningen, 1982). The B-subunits bind to ganglioside GMI on the cell membrane (van Heyningen, 1977) and this initial binding of the toxin must lead to the internalisation of the enzymically active A-subunit within the cell. The mechanism of entry is still not fully understood. The A2 chain is thought to be involved in the interaction of the A and B subunits, facilitating the entry of the enzymically active A1 peptide into the cell (van Heyningen, 1982). The A1 peptide has been compared to S-5 of pertussis toxin, however, on sequencing the genes no sequence homology was found (Nicosia *et al.*, 1986).

#### 1.4.2. *E. coli* heat-labile toxin

Certain strains of *E. coli* also secrete a toxin which activates adenylate cyclase. This protein is known as heat-labile toxin (LT) and causes diahorrea in the same way as cholera toxin. This toxin has the same subunit arrangement as cholera toxin (Clements *et al.*, 1980) with five B-subunits and one A-subunit which is made up of an A1 and an A2 peptide, the A1 peptide catalysing the ADP-ribosylation of an arginine

residue on Gs (Gill *et al.*, 1981; Moss and Vaughn, 1981). The DNA sequences of both cholera toxin and heat-labile toxin shows a significant identity of 95% (Splicer *et al.*, 1981) and they are thought to be derived from the same ancestral gene. LT has also been shown to bind to GM1 ganglioside (Parkinson and van Heyningen, 1989).

#### 1.4.3. Diphtheria toxin

Diphtheria toxin is synthesised by *Corneybacterium diphtheriae* and one molecule of this toxin is sufficient to cause death of a single cell. The toxin is synthesised as a single polypeptide chain which subsequently undergoes proteolytic cleavage into an A-fragment of 24 000 and a B-fragment of 38 000 molecular mass. The two fragments are held together by a single disulphide bond.

The receptor for this toxin is thought to be a cell-surface glycoprotein and the toxin binds to this receptor via the B-fragment, again resulting in the entry of the A-fragment into the cell. Once the A-fragment has dissociated from the B it catalyses the ADP-ribosylation of elongation factor 2 (EF-2). This ADP-ribosylation prevents protein synthesis and leads to cell-death (Honjo *et al.*, 1968). The residue which is modified by diphtheria toxin is diphthamide, a modified histidine residue which is only found in EF-2 (van Ness *et al.*, 1986).

#### 1.4.5. *Pseudomonas aeruginosa* exotoxin A

This toxin has the identical enzymic activity to diphtheria toxin. Both toxins catalysing the  $\text{NAD}^+$ -dependent ADP-ribosylation of elongation factor 2 (EF-2). Exotoxin A is also synthesised as a single precursor chain which is processed into A and B fragments. The mature fragments have molecular masses of 27 000 for A and 15 000 for B. As with the other toxins already discussed exotoxin A fragment B is responsible for receptor binding and A fragment internalisation (Iglewski and Kabat, 1975; Iglewski *et al.*, 1977). Although exotoxin A and diphtheria toxin have structural similarities they do not cross-react immunologically and do not bind to the same cell-surface receptor.

The three-dimensional structure of exotoxin A has been resolved by X-ray crystallographic techniques (Brandhuber *et al.*, 1988). The structure solved is the proenzymic form of the protein, ie. the form it takes before becoming internalised. The group have proposed a model for the interaction of  $\text{NAD}^+$  with exotoxin A which involves minor shifts, of less than one Å, in the backbone and position of the side chains upon binding of substrate. The nicotinamide ring of  $\text{NAD}^+$ , appears to stack on the whole ring of Trp 466, while the positively charged groups of Arg 456 and 467 form an ion bridge with the phosphates of  $\text{NAD}^+$ . Tyr 481 has been shown to interact with the purine ring of the adenine moiety of  $\text{NAD}^+$ . Tyr 470 participates directly in catalysis and is positioned near the nicotinamide-ribose bond which is broken down during catalysis. This proposed model is further confirmed by site-directed mutagenesis. Diphtheria toxin utilises similar catalytic residues and has a structurally similar binding site to that of exotoxin A so the X-ray structure data for exotoxin A may also apply to diphtheria toxin (Brandhuber *et al.*, 1988).

#### 1.4.5. The cytotoxins of *Clostridium botulinum*

Several of the clostridial bacteria produce toxins which ADP-ribosylate monomeric actin. These include botulinum C2 toxin, *Clostridium perfringens* iota toxin, *Clostridium spiroforme* toxin and a toxin produced by *Clostridium difficile*. These toxins, by ADP-ribosylating monomeric G-actin, inhibit polymerisation of the monomeric form by preventing further polymerisation at the end of actin filaments, resulting in the breakdown of the cytoskeleton (Aktories and Wegner, 1989).

C2 toxin is another two-component toxin, with component II being responsible for binding and component I being enzymically active. The mass of component II is 100 000 and of component I is 50 000. C2 toxin ADP-ribosylates non-muscle actin on Arg 177 (Vanderkerchove *et al.*, 1988). *C. perfringens* iota toxin modifies Arg 177 in skeletal muscle actin, so the specificity of ADP-ribosylation depends not only on the type of actin isoform but also on the toxin.

It has recently been shown that certain strains of *Clostridium botulinum* type C



produces a second ADP-ribosyltransferase, C3, which is distinct from toxin C2 (Aktories *et al.*, 1987). This toxin catalyses the ADP-ribosylation of a 21-24kDa GTP-binding protein found in various cell types. It has also been reported that toxin C1 and D ADP-ribosylate a 21kDa GTP-binding protein which is involved in the inhibition of exocytosis (Ohashi *et al.*, 1987) but Rosener *et al.*, (1987) have suggested that this observation is only as a result of contamination of preparations of C1 and D with toxin C3. This latter suggestion is now believed to be correct.

#### 1.4.6. The neurotoxins of *Clostridium botulinum*

*Clostridium botulinum* produces at least nine different toxins, types C2 and C3 being the cytotoxins discussed above and types A, B, C1, D, E, F and G being neurotoxins. The neurotoxins block the release of neurotransmitters at cholinergic and other synapses resulting in the flaccid paralysis which is characteristic of this disease (Habermann and Dreyer, 1986). All seven toxins have a similar general structure consisting of a heavy and light chain which are joined by a disulphide bond. The heavy chain is responsible for binding to a cell-surface receptor, but neither chain has been shown to be enzymically active.

#### 1.4.7. Tetanus toxin

Tetanus toxin is a neurotoxin secreted by *Clostridium tetani* and is responsible for the dramatic muscular spasms characteristic of the disease tetanus. Very little is known about the cellular or molecular events which result in the manifestation of this clinical disease. This is probably due to the site of action of this toxin being the central nervous system, which is itself very complex and not fully understood.

The toxin binds to peripheral nerve terminals, where it is internalised and transported by retrograde axonal transport to the cell body (Price *et al.*, 1975). The toxin migrates trans-synaptically until it reaches the inhibitory synapses of the spinal cord or brain stem. The toxin causes its spastic paralysis by blocking the release of neurotransmitters, especially gamma-aminobutyric acid (GABA), from these



inhibitory synapses. This inhibition of neurotransmitter release leads to the uninhibited firing of neurons and the characteristic spastic paralysis of tetanus.

The toxin itself is composed of a heavy chain of molecular mass 100 000 and a light chain of 50 000 molecular mass. Like diphtheria toxin, tetanus toxin is synthesised as a single polypeptide chain precursor, which is proteolytically nicked into heavy and light chains which are both held together by disulphide bonds (Craven and Dawson, 1973). Recent evidence has shown that the heavy chain is responsible for binding to a specific cell-surface receptor and micro-injection of the light chain into cells leads to inhibition of catecholamine release from adrenal chromaffin cells (Bittner and Holz, 1988; Penner et al., 1986).

TABLE 1.5 COMPARISON OF BACTERIAL A-B TYPE TOXINS

Toxin	Overall molecular mass	Structure	Mol. Mass.	Membrane target	Amino acid target	Structure	Mol. Mass	Target
Pertussis toxin	105-117000	S-1 subunit	28000	Inhibitory regulatory protein of adenylate cyclase-Gi and other GTP-binding proteins	Cysteine	Five B-subunits S-2, S-3, S-4 and S-5	S-2, 23 000 S-3 22 000 S-4 11 700 S-5 9 300	Receptor unknown evidence suggest a glycoprotein
Cholera toxin	82 000	A subunit A1 + A2 peptides	27000 total 22000 A1 5000 A2	Stimulatory regulatory protein of adenylate cyclase-Gs and other GTP-binding proteins	Arginine	Five B-subunits	5 x 11 600	Ganglioside GM1
<i>E. coli</i> heat labile toxin	91 000	A subunit A1 + A2 peptides	30000 total 25000 A1 5000 A2	Gs and other GTP-binding proteins	Arginine	Five B-subunits	5 x 11 800	Ganglioside GM1 and glycoproteins
Diphtheria toxin	62 000	A chain	24000	Elongation Factor 2	Diphthamide	B chain	38 000	Receptor unknown - thought to be a glycoprotein
<i>P. aeruginosa</i> exotoxin A	71 000	A fragment	27000	Elongation Factor 2	Diphthamide	B fragment	45 000	Unknown
<i>C. botulinum</i> type C2	150 000	Protein I	50000	G-actin filaments	Arginine	Protein II	100 000	Unknown
<i>C. botulinum</i> type C3			21-24KDa protein	Unknown				Unknown
Tetanus toxin	150 000	Light chain	50000	Function not well understood	Unknown	Heavy chain	100 000	Gangliosides GT1b and GD1b
Botulinum toxin	150 000	Light chain	50000	Function not well understood	Unknown	Heavy chain	100 000	Receptor unknown may be a glycolipid

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. MATERIALS

##### Radioactive chemicals

[Nicotinamide-4- $^3\text{H}$ ]NAD $^+$ , TRA.298, 925MBq (18.5-92.5GBq/mmol) and [carbonyl- $^{14}\text{C}$ ] NAD $^+$ , CAA 372, 370kBq (11 - 18.5GBq/mmol) were purchased from Amersham International, plc Amersham, Buckinghamshire, England.

[U- $^{14}\text{C}$ ]cysteine and [adenine-2-8  $^3\text{H}$ ]NAD $^+$  were from Dupont (U.K.) Ltd., Wedgewood Way, Stevenage, Hertfordshire, England.

“Optiphase Safe” scintillation fluid was from Pharmacia, LKB Biotechnology, S-75182, Uppsala, Sweden.

##### Other materials and reagents

Adenosine 5'-diphosphoribose (ADP-ribose), Adenosine 5'-monophosphate (AMP), Bovine serum albumin, 4-chloro-1-naphthol, L-cysteine methyl ester (hydrochloride), dimethyl suberimide, 1-ethyl-3-[dimethyl-amino propyl] butyric acid N-hydrosuccinimide ester, snake venom phosphodiesterase and ovalbumin were from Sigma Chemical Co., Poole, Dorset, England.

NAD $^+$  (Grade 1, 100%) was from Boehringer Mannheim, (UK), Bell Lane, Lewes, East Sussex, England.

Silver nitrate and other analytical reagents were from B.D.H. Chemicals,

Poole, Dorset, England.

Dithobis (succinimidyl propionate) (DSP) and disuccinimidyl tartarate (DST) were from Pierce Chemical Co., P.O. Box 1512, 3260BA Oud-Beijerland, The Netherlands.

Cyanogen bromide activated sepharose 4B was from Pharmacia LKB Biotechnology, S-75182, Uppsala, Sweden.

Dowex AG-1X (200-400 mesh, chloride form) was purchased from Bio Rad, Watford, Hertfordshire, England.

Spectrapor dialysis membrane was from Spectrum Medical Industries, 60916 Terminal Annex, Los Angeles, U.S.A.

[12-[(phenyl methyl) amino]dodecanyl]guanidine (A599) and [10- (acetyl amino) decanyl] guanidine (A603) were made available as intermediates from synthetic programmes within the Glaxo Research Group Ltd. *Wellwyn Garden City, London, U.K.*

All other reagents were analytical grade.

## 2.2 METHODS

### 2.2.1 Preparation of pertussis toxin - general method

Pertussis toxin was purified from culture supernatants of *Bordetella pertussis*. The method followed was that of Irons and MacLennan (1979) and Sekura *et al.* (1983). The following flow diagram outlines the general method of toxin purification.

Freeze-dried *B. pertussis* (Wellcome strain 28), 0.5ml, is resuspended in H<sub>2</sub>O and growth is initiated on charcoal



↓  
agar plates (oxoid CM119) containing 10% w/v defibrinated horse blood.

↓  
Growth from these plates is used to inoculate seed cultures containing 100ml of CL+CD medium (discussed in Chapter 3). Seed cultures are grown at 35°C for 24 hours.

↓  
9ml of seed culture is used to inoculate Thompson flasks containing 300ml of medium. The cultures were grown for 48-58 hours when bacterial growth was at its maximum. The O.D. of the culture must be >6 which corresponds approximately to 2.5mg dry weight of bacteria per ml.

↓  
Cultures centrifuged for 75 mins at 5000rpm  
Supernatant removed and filtered through 0.22μ filter to remove all contaminating cells.

↓  
Culture supernatant is then passed through a hydroxyl apatite column. This column binds filamentous haemagglutinin, the other major secreted protein of *B. pertussis* and allows pertussis toxin to pass straight through.

↓  
The eluate is precipitated with  $(\text{NH}_4)_2\text{SO}_4$  to a final concentration of 74% saturation

↓  
The ammonium sulphate precipitated protein is centrifuged at 4000 rpm for 1 hour, the supernatant removed and the pellet resuspended in 0.05M phosphate, 50mM NaCl buffer, pH 7.2. The suspension is centrifuged at 15000 rpm for 20 minutes, supernatant decanted and the pellet extracted four times with phos/NaCl buffer. The supernatants are combined and dialysed overnight.

↓  
The dialysate is then applied to fetuin-Sepharose affinity column.

↓

↓

Pertussis toxin binds specifically to this column and is eluted with 6.7mM tris, 0.13M NaCl, 3M MgCl, pH. 6.4 buffer.

40 x 50 drop fractions are collected and each fraction measured for its haemagglutinin activity with goose erythrocytes.

↓

Those fractions with haemagglutinin titre >7 are pooled and measured for protein content.

The purified pertussis toxin is run on an SDS polyacrylamide gel to confirm the homogeneity of the preparation.

## **2.2.2. ELECTROPHORETIC TECHNIQUES**

### **2.2.2.1 SDS polyacrylamide gel electrophoresis (P.A.G.E.) general method**

Polyacrylamide gel electrophoresis was routinely carried out under denaturing conditions by the addition of 0.1% (w/v) SDS in both the separating and stacking gels. The gel system was based upon that of Laemmli (1970) with the addition of 2mM EDTA. to chelate metal ions which may interfere with the polymerisation of acrylamide and cause aggregation of proteins (Douglas and Butow, 1976). Protein samples were electrophoresed for 1 hour at 100 volts while the proteins moved into the stacking gel and then at 75 volts overnight.

Gels were fixed in 20% (v/v) methanol, 10% (v/v) acetic acid for 15 minutes, stained in 0.25% (w/v) Coomassie brilliant blue R-250, 45% (v/v) methanol, 9% (v/v) acetic acid for 15 minutes at 55°C and destained in methanol 5% (v/v), 7.5% (v/v) acetic acid at 55°C until the background was clear. The addition of polystyrene quickened the destaining process by absorbing the dye.

For determination of molecular masses, gels were calibrated with the following standard proteins: bovine milk  $\alpha$ -lactalbumin (14.2kDa), soybean

trypsin-inhibitor (20.1kDa), bovine pancreas trypsinogen (24kDa), bovine erythrocyte carbonic anhydrase (29kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36kDa), rabbit phosphorylase b (subunit) (97.4kDa), *E. coli*  $\beta$ -galactosidase (subunit) 116kDa and rabbit muscle myosin (subunit) 205kDa. These proteins were supplied as kits from Sigma Chemical Co: Dalton Mark VII-L<sup>TM</sup> molecular mass marker kit for determination of molecular masses in the range 14.2kDa to 66kDa and the higher molecular mass standard mixture for determination of molecular masses in the range 29kDa up to 205kDa.

#### **2.2.2.2. Gradient polyacrylamide gel electrophoresis**

Gradient polyacrylamide gel electrophoresis was carried out essentially as outlined above. 5 - 20% (w/v) acrylamide and 10 - 20% (w/v) acrylamide gradient polyacrylamide gels were poured using a Biorad 305 model gradient former. Protein samples were again electrophoresed at 100 volts, while the proteins moved into the stacking gel and then at 75 volts overnight. The gels were fixed, stained and destained as outlined above.

#### **2.2.2.3. Western blotting**

Proteins were separated by SDS PAGE and transferred onto nitrocellulose essentially by the method of Towbin et al., (1979). Following transfer, unreacted binding sites were blocked by a 2 hour incubation in 0.3% (v/v) Tween 20, 1% (w/v) BSA in phosphate buffered saline pH. 7.2. The nitrocellulose was then washed with 5ml 0.3% Tween 20 in PBS for 3 x 5 minutes. The nitrocellulose was next incubated for two hours with a solution of first antibody, consisting of a 1:50 dilution of ascites fluid containing polyclonal antibody directed against one of the pertussis toxin subunits. The strips were again washed in 5ml of PBS/Tween 20 for 3 x 5 minutes after which time second antibody, the anti-mouse, horse-raddish peroxidase conjugate, was added and the strips incubated for a further 2 hours.

The strips were washed for a final 3 x 5 minutes in PBS and the bands were visualised with 4-chloro-1-naphthol (0.02% w/v) dissolved in 1ml of methanol and made up to 50ml with PBS. This was added to the washed strips with 10 $\mu$ l of H<sub>2</sub>O<sub>2</sub>. The bands generally took 5-10 minutes to develop and the reaction was stopped by immersing the strips in H<sub>2</sub>O.

#### **2.2.2.4. Silver staining**

The method used was essentially that of (Wary *et al.*, 1981). 0.8g of silver nitrate was dissolved in 4ml of H<sub>2</sub>O and added dropwise, on stirring, to 21 ml of 0.36% (w/v) NaOH and 1.4ml of concentrated NH<sub>3</sub> solution. This solution was made up to 100ml with H<sub>2</sub>O. The SDS polyacrylamide gel which had been soaked overnight in 50% MeOH was soaked in this solution for 15 minutes. After the soaking period the gel was washed for 3 x 5 minutes with distilled H<sub>2</sub>O and developed in 500ml of 0.5% (v/v) formaldehyde, 1% (v/v) acetic acid solution. The gel was generally developed in 5 minutes and development was stopped in 25% (v/v) isopropanol, 10% (v/v) acetic acid solution.

#### **2.2.2.5. FPLC separation of pertussis toxin subunits**

2ml of 3-4mg/ml ammonium sulphate suspension of pertussis toxin was pelleted at 13000 rpm in an MSE microcentaur and the protein resuspended in 10ml of 6M guanidinium chloride in 50mM sodium phosphate buffer, pH. 6.5 and stored at room temperature overnight. The sample was then extensively dialysed against 500ml of 8M urea in phosphate buffer for two days with frequent buffer changes to ensure the removal of the guanidinium chloride. The sample was applied to a Superose 12 FPLC column which had been pre-equilibrated in 50mM sodium phosphate, 0.5M NaCl, 8M urea pH 7.0 buffer. The column was eluted with the same buffer at a flow rate of 0.2ml/min. 50 x 1 ml fractions were collected and those fractions containing protein, as shown by their absorbance at 280nm, were analysed by SDS PAGE.



### 2.2.3. NAD<sup>+</sup> STUDIES

#### Assay of NAD<sup>+</sup>-glycohydrolase - general method

The NAD<sup>+</sup>-glycohydrolase activity of pertussis toxin was estimated indirectly by measuring the rate of [<sup>3</sup>H]-nicotinamide release from [4-<sup>3</sup>H] NAD<sup>+</sup> by the method of Moss and Vaughn (1984). The assay <sup>mixture</sup> contained 20mM potassium phosphate, pH. 6.5, 250mM dithiothreitol, 1mg/ml ovalbumin, concentrations of NAD<sup>+</sup> ranging from 1μM - 200μM containing 20μCi [4-<sup>3</sup>H] NAD<sup>+</sup> and 10μg of pre-activated toxin, in a total volume of 300μl. The assays were initiated by the addition of pre-activated pertussis toxin to the remaining constituents and incubated for 4 hours at 30°C. 0.1 ml of products were applied to columns (0.5 x 2cm) of Dowex AG-1X resin (200 - 400 mesh, chloride form, supplied by Bio Rad). The columns were previously washed twice with 1ml of H<sub>2</sub>O. [<sup>3</sup>H] nicotinamide was eluted with five 1ml portions of 20mM tris, pH. 7.5 buffer and 1 ml sample of the eluate added to 5ml of scintillation fluid and counted for radioactivity. The pmoles of nicotinamide produced per hour can then be calculated.

#### 2.2.3.2. Activation of pertussis toxin

Pertussis toxin was activated prior to its use in the NAD<sup>+</sup>-glycohydrolase assay by reduction of <sup>the</sup> disulphide bond in the S-1 subunit. The activation buffer consisted of 200mM potassium phosphate pH. 6.5; 20mM dithiothreitol and 1% (w/v) BSA. Toxin was activated in this buffer for 30 minutes at 30°C.

#### **2.2.3.3. Determination of the efficiency of NAD<sup>+</sup>-glycohydrolase**

The efficiency of the Dowex AG-1X2 separation of NAD<sup>+</sup> and nicotinamide was demonstrated by paper chromatographic separation of the NAD<sup>+</sup>-glycohydrolase reaction products and comparing this separation with the resolution of [4 <sup>3</sup>H] NAD<sup>+</sup> and [<sup>3</sup>H] nicotinamide standard solutions. A mixture of 1μCi [4 <sup>3</sup>H] NAD<sup>+</sup>: 1μCi [<sup>3</sup>H] nicotinamide was made up in 200mM phosphate buffer, pH. 6.5 to a total volume of 300μl. 100μl of this mixture was added to two columns, the remaining 100μl being used as a control. The columns were washed with 5ml of 20mM Tris, the control sample being diluted to a final volume of 5ml, and 20μl of each applied to a strip of Whatman 3mm chromatography paper.. The reaction products were separated in a mobile phase of isobutyric acid: NH<sub>4</sub>OH: H<sub>2</sub>O in the ratio 66:1:33 for 4 hours. The strips were then dried and cut into 1cm x 2cm portions and placed in scintillation vials containing 1ml of H<sub>2</sub>O and washed for 30 minutes. The strips were then removed and 10ml of scintillation fluid added to the vials. Each sample was counted for <sup>3</sup>H activity.

#### **2.2.3.4. Sulphydryl reagents as potential substrates**

The requirement for such a large dithiothreitol concentration within the NAD<sup>+</sup>-glycohydrolase assay mixture was investigated by essentially the same method as outlined in section 2.2.3.1. The rate of [<sup>3</sup>H] nicotinamide release from [4 <sup>3</sup>H] NAD<sup>+</sup> was again determined, only in this case at saturating levels of NAD<sup>+</sup> (100μM) and with dithiothreitol concentrations ranging from 50mM - 1M. The remaining assay constituents were as before. The pmoles of nicotinamide released per hour were again calculated. This procedure was repeated using the sulphydryl

reagent cysteine,  $\beta$ -mercaptoethanol and glutathione.

#### **2.2.3.5. HPLC separation of NAD<sup>+</sup>-glycohydrolase reaction products**

The products of the NAD<sup>+</sup>-glycohydrolase reaction, as outlined in section 2.2.3.1., were separated by HPLC. The reaction products were firstly filtered through an Amicon OM-10 filter, to remove all protein, and then the sample applied to a reverse-phase ODS HYP 2734 column, dimensions 25cm x 4.6cm. The mobile phase was 90% (v/v) 10mM ammonium dihydrogen phosphate, pH. 3.5 10% (v/v) methanol run at a flow rate of 1ml/min. The chart recorder was set at 1cm/min. The absorbance of the eluate was measured at 254nm using Altex equipment.

The system was characterised using ADP-ribose, NAD<sup>+</sup>, AMP and nicotinamide as standards enabling most of the reaction products to be identified.

#### **2.2.3.6. Identification of reaction products using radioactive compounds**

Products of the NAD<sup>+</sup>-glycohydrolase reaction were separated by reverse-phase HPLC as previously described, only in this case the assay mixture contained [adenine-2-8 <sup>3</sup>H] NAD<sup>+</sup> (2.5 $\mu$ Ci/ $\mu$ mol) and [U-<sup>14</sup>C] cysteine (1.3 $\mu$ Ci/mmol). The eluate was collected as 90 x 5 drop fractions directly into scintillation vials and each fraction counted for both [<sup>3</sup>H] and [<sup>14</sup>C] radioactivities. The peaks of radioactivity were matched with their corresponding peaks on the HPLC trace. In the case of cysteine, which does not absorb at 254nm, a standard solution of [U-<sup>14</sup>C] cysteine was applied to the column and 90 x 5 drop fractions collected, each fraction being

counted for  $^{14}\text{C}$  radioactivity. From this run the elution position of cysteine could be plotted on the trace.

#### **2.2.3.7. Isolation of putative ADP-ribosyl-cysteine**

From the elution time of this reaction product, the fractions containing this compound were collected and concentrated by freeze-drying. The isolated product was run on the HPLC at high sensitivity to demonstrate the homogeneity of the preparations.

#### **2.2.3.8. Reaction of snake-venom phosphodiesterase**

The isolated reaction product was incubated with snake-venom phosphodiesterase for 5 hours at  $37^{\circ}\text{C}$ . The products of this reaction were filtered through an Amicon OM-10 filter to remove all protein and the sample applied to the HPLC column to identify the reaction products.

#### **2.2.3.9. Equilibrium dialysis**

These studies were carried out in Teflon cells (Dianorm "R", Fisons Ltd). Each of the cell consists of two teflon half-cells of total volume  $250\mu\text{l}$  which are separated by a dialysis membrane (Spectrapor) of molecular weight cut-off 6 000 - 8 000. Five such cells are arranged in an assembly which is gently rotated in a waterbath at constant temperature. The buffer used for binding experiments was 0.1M sodium chloride, 10mM potassium phosphate, pH. 7.5, 1M urea. Buffer containing  $[4\text{-}^3\text{H}] \text{NAD}^+$  ( $20\mu\text{Ci}$ ) and concentrations of unlabelled  $\text{NAD}^+$  from  $1\mu\text{M}$  up to  $200\mu\text{M}$  in a total volume of  $200\mu\text{l}$  was injected into one half-cell. Pertussis toxin at concentrations of 1.4-1.7 mg/ml, depending upon experiment, was also added to this buffer in a total volume of  $200\mu\text{l}$  and injected into the other half-cell.

Several preliminary experiments were carried out in order to characterise the system. The time taken to reach equilibrium was determined in a toxin-free system and shown to be 3 hours. The non-specific breakdown of  $\text{NAD}^+$  to ADP-ribose and nicotinamide during the dialysis procedure was found to be negligible and finally the overall recovery of radioactivity was shown to be >95% in all cases.

#### 2.2.3.10. Fluorescence measurements

Fluorescence titrations were carried out in a Perkin-Elmer MPF-3000 spectrofluorimeter containing a thermostatically controlled cuvette holder. The excitation and emission slits were set at 15nm and 5nm respectively. Titrations were performed by adding 5-10 $\mu\text{l}$  increments of  $\text{NAD}^+$  <sup>10mM</sup> in potassium phosphate, 0.1M sodium chloride, pH. 7.5 buffer to a 2ml solution of 0.1mg/ml pertussis toxin in the same buffer. The samples were carefully stirred after each addition, ensuring that light was always excluded from the system. The decline in protein fluorescence was measured at 340nm emission, 280nm excitation. The temperature of the samples was kept constant at 23°C, 30°C or 40°C. In order to correct for inner filter effects a solution of pertussis toxin, at the same concentration as used for fluorescence titrations, was similarly titrated in a Perkin-Elmer spectrometer at 280nm and corrections were made according to Honore and Pederson (1989). All data were corrected for dilution.

#### 2.2.3.11. Circular dichroism of pertussis toxin

To ensure that 1M urea had no effect upon the tertiary structure of pertussis toxin, and thus <sup>would</sup> have no effect upon the binding of  $\text{NAD}^+$ , circular dichroism spectra were obtained in the presence and absence of urea. This work was carried out at CAMR, Porton Down. The appropriate buffer blanks were subtracted in each case and scans were carried out in the near U.V. (300 - 250nm) and in the far

U.V. (250 - 200 nm). All spectra expressed as mean <sup>molar</sup> residue ellipticity  $[\theta]$  as degree cm<sup>2</sup>/dmol against wavelength (nm).

$$\phi = \frac{\text{MRW}}{100} \times \frac{\Theta}{\text{LC}} \quad 1.$$

$\Theta$  = measured ellipticity

L = cell pathlength in dm

C = concentration in g/ml (measured by micro-Kjeldahl) (Morrison and Boyd, 1974)

MRW = mean residue weight = molecular mass / total number of amino acids  
for pertussis MRW = 110

#### 2.2.4. Cross-linking studies

##### 2.2.4.1. Cross-linking of pertussis toxin subunits

An ammonium sulphate suspension of pertussis toxin containing 5mg of pertussis toxin was pelleted by centrifugation at 13 000 rpm in an MSE microcentaur and resuspended in 5ml of 0.1M potassium phosphate, 0.5M NaCl pH. 7.5 buffer and dialysed against this buffer to remove all contaminating ammonium sulphate. Cross-linking reagents were dissolved in DMSO and added to aliquots of the 1mg/ml toxin solution to a final concentration of 1mM or 10mM. The reaction mixture was left at room temperature for 4 hours with constant mixing after which time SDS was added to a final concentration of 1% (w/v) and the samples boiled for 5 minutes. After boiling, the samples were applied to a 5% - 20% (w/v) or 10% - 20% (w/v) gradient acrylamide SDS polyacrylamide gel.

##### 2.2.4.2. Two-dimensional SDS-polyacrylamide gel electrophoresis

Cross-linked pertussis toxin subunits were separated on a 1st-dimension SDS polyacrylamide gel as described above. These gels consisted of acrylamide

gradients of 5-20% (w/v) acrylamide or 10-20% (w/v) acrylamide. Strips were cut from these gels and treated in one of the following ways.

1. The excised strip was soaked in a solution of 0.1M tris, 10mM dithiothreitol pH. 6.8 buffer for 2 hours. The strip was then attached to the top of a second dimension 17.5% polyacrylamide gel with agarose and the proteins run into the separating gel as before.
2. The excised strip was attached to a 17.5% separating gel with agarose and the cross-linked subunits allowed to penetrate the separating gel which contained 10mM dithiothreitol. Once the cross-linked subunits had penetrated this gel, the current was switched off for 2 hours, after which time the current was reapplied and the gel run to completion.
3. The final method was a modification of (2) in which 10mM dithiothreitol was only incorporated within the stacking gel. The cross-linked subunits from the 1st dimension are allowed to penetrate the stacking gel, the current then being turned off for 2 hours, reapplied, and the gel run to completion.

#### 2.2.4.3. Electroelution

Strips of polyacrylamide gel containing protein were removed from the gel and cut into 10 portions each 10x2cm. These were positioned in the large cup of the Isoo electroelution apparatus. The buffer used was 10mM potassium phosphate pH 7.5, 1M urea. Application of current results in proteins migrating from the gel matrix to the concentrating cup which holds a total volume of 50µl.

Isoo, Inc. P.O. Box 5347, Lincoln, NE 68505, USA.



#### **2.2.5. Determination of protein**

Unless otherwise stated, protein was measured using the technique of Lowry, et al., (1951). At CAMR the method used is an automated Lowry which is calibrated with bovine serum albumin.

#### **2.2.6. Liquid scintillation counting**

All radioactive samples were counted in "Optiphase Safe" emulsifying scintillant from LKB. For each experiment all samples were identical in volume of scintillant and volume of sample. Background and standard vials were also counted for each experiment. The background vials were identical in both sample volume and volume of scintillant as the sample vials, except that they contained no radioactive material. The standard vials contained a known amount of sample containing a defined amount of radioactivity.



## CHAPTER 3

### GROWTH OF *BORDETELLA PERTUSSIS* AND PURIFICATION OF PERTUSSIS TOXIN

#### 3.1. INTRODUCTION

##### 3.1.1. Expression of pertussis toxin

Pertussis toxin is found in both the supernatants and cell pellets of *B. pertussis* (Morse and Bray, 1969, Sato, et al., 1974). Ashworth, et al., (1985) showed, by electron microscopy using a gold-labelled monoclonal antibody, that the toxin was distributed all over the cell surface. Little is known on how the toxin is released into culture supernatants. Evidence suggests that this secretion mechanism does not involve the autolysis of cells (Sato, et al., 1974; Perera, et al., 1986) as it has been shown that malate dehydrogenase, a cytoplasmic marker, does not appear in culture supernatants. Loch and Keith, (1986) and Nicosia et al., (1986) have both shown that each of the pertussis toxin subunits contains a region at its amino terminal which is typical of a bacterial signal peptide. This suggests that each of the subunits are synthesised individually as precursor proteins whereupon they are secreted into the periplasmic space, their signal peptides are subsequently cleaved and the mature subunits assembled into the holotoxin molecule.

##### 3.1.2. Growth of *Bordetella pertussis*

Maximum concentration of pertussis toxin is found at the end of the exponential growth phase and the beginning of the stationary phase (Sato, et al., 1974; Sekura, et al., 1983; Bellalou and Relyveld, 1984). A batch culture process is used in the purification of pertussis toxin as Gorringe, (1988) showed that a greater amount of pertussis toxin was obtained from batch culture (Stanier and Scholte, 1971), when compared to continuous culture (Robinson, et al., 1983). This is easily explained as

continuous culture steady-state conditions are maintained in the mid-exponential phase where production of pertussis toxin is not maximum.

### 3.1.3 Growth of *Bordetella pertussis*

#### 3.1.3.1. Initiation of Growth

This work was kindly carried out by Dr. Andrew Gorringer, Vaccine Research and Production Laboratory, P.H.L.S. Centre for Applied Microbiology and Research, Porton Down.

Growth of *B. pertussis* strain Wellcome 28 was initiated by resuspending 0.5ml of the bacteria, which are freeze-dried in medium, and growing the resuspended cultures on charcoal agar plates (oxoid CM119) which contain defibrinated horse blood (10% v/v). The resuspended bacteria were spread onto the surface of two charcoal agar plates and the cultures grown at 35°C for 48 hours, after which time the growth from the plates were used to inoculate seed cultures. These seed cultures are grown in 100ml of CL + CD medium for 24 hours at 35°. After this incubation period 9ml of seed culture was used to inoculate thirty 2.5 litre Thompson flasks (25cm x 15cm x 7.5cm).

#### 3.1.3.2. Growth medium

The strain of *Bordetella pertussis* used for purification of pertussis toxin is strain Wellcome 28. The cultures are grown in Thompson bottles, each containing 300ml of medium, and incubated at 35°C on a shaker. 2,6-O dimethyl  $\beta$ -cyclodextrin (MeCD) is added to the medium as it has been shown to stimulate production of pertussis toxin by about two-fold (Imaizumi, et al., 1983a and b). It is added to CL+CD medium to a final concentration of 1g/l

### 3.1.3.3. Preparation of CL and CD medium

The additions for CL + CD medium are prepared as follows:

- (a) 5g L-cysteine in 400ml 2.5M HCl
- (b) 1.0g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  in 400ml of distilled  $\text{H}_2\text{O}$
- (c) 0.4g nicotinic acid in 400ml of distilled  $\text{H}_2\text{O}$

The bottle for medium preparation is filled with 10 litres of distilled water and the following medium constituents are added:

	<u>g/20 litres</u>
Sodium glutamate	214.0
L-proline	4.8
NaCl	50.0
$\text{KH}_2\text{PO}_4$	10.0
KCl	4.0
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	2.0
$\text{CaCl}_2$	0.4
Tris	122.0
Glutathione (reduced)	3.0
Ascorbic acid	8.0
Casamino acids	200.0
MeCD	20.0

80ml of the cysteine/ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /nicotinic acid solution is then added to the 10 litres of medium and the total volume of medium is made up to 20 litres with distilled water. The pH of the medium is then adjusted to 7.6 with HCl. 300ml of medium is added to each Thompson bottle, with 30 Thompsons generally being used in one toxin purification process.

The Thompson bottles are incubated at 35°C for 24 hours before inoculation, during which time any of the bottles which show signs of bacterial growth are

discarded. 9ml of seed culture are then used to inoculate each of the Thompson fasks and these are again incubated at 35°C with shaking. Growth in the Thomspson flasks is usually complete within 48-58 hours. This is confirmed by determination of the optical density of the bacterial cultures at 550nm. When the O.D. 550nm is greater than 6 then growth is complete as this optical density corresponds to approximately 2.5mg dry weight of bacteria per millilitre of culture.

### **3.2. Purification of pertussis toxin**

#### **3.2.1 Introduction**

Some of the properties of pertussis toxin complicate its purification process. These are: firstly its increased insolubility in low ionic strength buffers as the purification process proceeds, secondly that the toxin is inactivated by organic solvents and by exposure to pH less than 3 and greater than 9, and finally that it is strongly adsorbed to many of the routinely used chromatographic media.

Sato and Arai, (1972) and Sato, et al., (1974) were first to purify pertussis toxin. They used three consecutive steps of ammonium sulphate precipitation, followed by starch-block electrophoresis and finally sucrose density gradient. However, this method of toxin purification did not separate the two major haemagglutinins secreted by the bacteria, namely pertussis toxin and filamentous haemagglutinin. Yajima, et al., (1978a) purified the toxin to homogeneity by firstly passing the culture supernatant down a hydroxyl apatite column, then a Concanavalin-A-Sepharose column and finally a Biogel P-100 column. They called their purified protein islet-activating protein as it was shown to enhance insulin secretion for the  $\beta$ -cells of the pancreas. Their purified toxin was also shown to have lymphocytosis promoting and histamine-sensitising activities in mice (Yajima, et al., 1978b).

In 1979 Irons and MacLennan showed that pertussis toxin could be purified by a one-step affinity chromatographic process. This was based on the observation that pertussis toxin specifically bound to serum sialoglycoproteins. The affinity matrix used

was human haptoglobulin coupled to Sepharose 4B. Pertussis toxin specifically bound to this matrix and was eluted by a step-wise pH gradient. This method was then modified by Sekura *et al.*, (1983) who coupled fetuin to Sepharose 4B as it was a more commercially available glycoprotein. Sato, *et al.*, (1983) carried out a two-step chromatography purification process firstly involving hydroxylapatite chromatography and secondly haptoglobulin-Sepharose chromatography. This method enabled a good separation of pertussis toxin from filamentous haemagglutinin at the hydroxylapatite stage leading to a very pure toxin preparation. This affinity chromatography method is the method routinely carried out at CAMR.

### 3.2.2 PURIFICATION OF PERTUSSIS TOXIN

#### 3.2.2.1 Hydroxyl apatite chromatography

Once the O.D. of the bacterial culture has reached its maximum the 30 litres of culture was centrifuged at 5 000 rpm for 75 minutes whereupon the culture supernatant was removed. The supernatant was then filtered through a 0.22 $\mu$  filter to remove all contaminating cells. At this stage of the purification process the supernatant is mainly composed of pertussis toxin and filamentous haemagglutinin. The filtered supernatant was then pumped through a hydroxyl apatite column.

The spherical hydroxyl apatite used was supplied by BDH Chemicals Ltd. 400g of this powder was suspended in 500ml of 0.1M NaCl and the mixture allowed to settle at room temperature. The above procedure was repeated a further twice after which the hydroxyl apatite is washed with several washes of distilled water until the pH of the decanted solution is 8. The powder was then resuspended in 10mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer, p.H. 8, decanted and washed with a further three volumes of this buffer. The hydroxyl apatite was suspended in this buffer and poured into the column. The column was further washed with phosphate buffer for 7 hours at a flow rate of 400 ml/hr and overnight at 200 ml/hr. This extensive washing is required to ensure that the final bicarbonate concentration is less than 0.2%. The column was then ready for use.

The column was connected to a reservoir containing the concentrated filtered

culture supernatants and the solution is pumped through the column at a flow rate of 200 ml/hr. The eluate is collected from the column and contains pertussis toxin, filamentous haemagglutinin remaining bound to the column. Ammonium sulphate (Aristar grade) is added to the eluate to a final concentration of 74% (50g of  $[\text{NH}_4]_2\text{SO}_4$  per 100 ml of eluate) and this is left overnight to precipitate pertussis toxin. The precipitated eluate is then centrifuged at 4000 rpm for 60 minutes at 4°C. The pellet is resuspended in 0.05 M phosphate, 0.5 M NaCl buffer, pH. 7.2 and this suspension is further centrifuged at 15 000 rpm for 20 minutes. The supernatant is decanted and the pellet extracted four times with the phos/NaCl buffer. A hand homogeniser is used each time to resuspend the pellet and ensure a homogenous suspension. The supernatants are combined and dialysed overnight against phos/NaCl buffer at 4°C. The dialysed sample is then applied to a fetuin-Sepharose affinity column which has been pre-equilibrated with the dialysis buffer.

#### **3.2.2.2. Preparation of fetuin-Sepharose 4B**

15 g of CNBr-activated Sepharose 4B is suspended in 100 ml of 1M HCl and applied to a scintered glass funnel (coarse grade 1 or 2) and washed with 2 litres of 1 M HCl. 200mg of fetuin is then dissolved in 10 ml of 0.1 M  $\text{NaHCO}_3$ , 0.5 M NaCl, pH 8.1 buffer and dialysed against this buffer for 16 hours at room temperature. The fetuin solution is next added to the washed Sepharose with 10 ml of 0.1 M  $\text{NaHCO}_3$ , 0.5 M NaCl, pH 8.1 buffer. This suspension is then rotated end over end for 4 hours at room temperature (the use of a magnetic stirrer was not advisable as it breaks up the Sepharose beads). The suspension is then filtered, the filtrate being collected for Lowry protein determination. The protein content of the filtrate should be low (<50µg/ml) showing that most of the fetuin has bound to the Sepharose. The Sepharose is then washed with a further 50 ml of  $\text{NaHCO}_3$ /NaCl buffer. This suspension is filtered and the Sepharose resuspended in 100 ml of 1 M ethanolamine, 0.1 M borate, 0.5 M NaCl, p.H. 9.0 buffer and left to equilibrate at room temperature for 2 hours. This suspension is again filtered and washed with four alternate washes of 0.1 M borate, 0.5 M NaCl,

p.H. 8.1 buffer and 0.1 M acetate, 0.5 M NaCl buffer, p.H. 3.8. The Sepharose is now ready for use.

### 3.2.2.3. Fetuin-Sepharose 4B affinity chromatography

The dialysed sample is applied to the fetuin-Sepharose column which has been pre-equilibrated with the dialysis buffer. The protein solution is loaded onto the columns at a flow rate of 0.5ml/min and the eluate collected. The column is then eluted with 50 ml of phosphate/NaCl buffer to wash off non-specific adsorbed components. The column is further eluted with this buffer collecting 10 x 50 drop fractions (3 ml). The column is then eluted with 6.7 mM tris, 0.13 M NaCl, 3 M MgCl p.H. 6.4 buffer at a flow rate of 0.5 ml/min. 40 x 50 drop fractions were collected and those containing pertussis toxin were determined via their haemagglutinin titre with goose erythrocytes.

## 3.3. RESULTS

### 3.3.1. Growth of *Bordetella pertussis*

Two consecutive cultures of *B. pertussis* were grown for the preparation of pertussis toxin as outlined in 3.1.2. After 48 hours of growth in Thompson flasks the optical density and p.H. of selected cultures were measured and the results for both preparations - ML-1 and ML-2 are shown in Table 3.1. The cultures were ready for centrifugation as the desired optical density was greater than 8 and the p.H. of the media 8.4.

The culture supernatant is prepared for fetuin-Sepharose 4B chromatography by the consecutive steps of filtration, hydroxylapatite chromatography and ammonium sulphate precipitation as shown previously. 40 fractions each 50 drops were collected and tested for their haemagglutinin titre.



### 3.3.2. HAEMAGGLUTININ TEST

#### 3.3.2.1. Preparation of goose red blood cells

20 ml of fresh goose blood was added to a glass centrifuge tube, which was filled to three quarters full with PBS. This suspension was centrifuged at 1,500 rpm for 5 minutes, the supernatant removed, and the pellet of red blood cells resuspended in PBS. This process was repeated for a further two washes. After the final wash, 0.5 ml of the packed red blood cells were resuspended in 100 ml of PBS. to a final concentration of 0.5% (v/v) of washed goose red blood cells.

**TABLE 3.1**            **Optical Density and p.H. of Selected *B. pertussis* cultures.**

Sample	Optical Density	p.H
ML-1 Sample 1	0.818	8.69
ML-1 Sample 2	0.862	8.50
ML-1 Sample 3	0.772	8.21
ML-2 Sample 1	8.08	8.32
ML-2 Sample 2	8.33	8.27
ML-2 Sample 3	7.97	8.33



### 3.3.2.2. Haemagglutinin test

50 $\mu$ l of PBS was added to each well of a microtitre tray using an eight channel automatic pipette. 50 $\mu$ l of each sample from the fetuin-Sepharose column was then added to duplicate wells on the tray and diluted through the row, leaving two rows of wells with no sample for control purposes. 50 $\mu$ l of freshly washed 0.5% (v/v) goose red-blood cells were then added to each well and the blood cells allowed to settle until "clear buttons" of red blood cells have formed in the control wells. The number of doubling dilutions of each fraction where there was no button formation in the well was then counted - the "haemagglutination titre" of each fraction. The titre of each fraction was recorded and those fractions of titre greater than 7 are pooled and assayed for protein content by the method of Lowry *et al.* (1951). The results for preparations ML-1 and ML-2 are shown in Table 3.2. The average protein content of preparation ML-1 is 0.6 mg/ml and of preparation ML-2, 0.8 mg/ml. Both samples were run on an SDS polyacrylamide gel, the gel is shown in Figure 3.1. The gel shows that all of the toxin subunits are intact. The toxin was stored as an ammonium sulphate suspension. The final protein concentrations of the preparations after ammonium sulphate precipitation are ML-1, 3.2 mg of toxin per ml of solution and ML-2, 3.5 mg of protein per ml as measured by the method of Lowry *et al.* (1951).

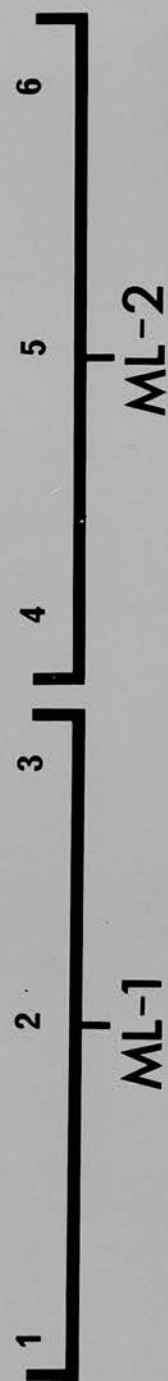
### **FIGURE 3.1**

**17.5% (w/v) acrylamide SDS polyacrylamide gel of pertussis toxin showing that all five subunits are intact in both ML-1 and ML-2 preparations.**

<b>Lane 1</b>	<b>50<math>\mu</math>l of ML-1 preparation</b>
<b>Lane 2</b>	<b>30<math>\mu</math>l of ML-1 preparation</b>
<b>Lane 3</b>	<b>10<math>\mu</math>l of ML-1 preparation</b>
<b>Lane 4</b>	<b>10<math>\mu</math>l of ML-2 preparation</b>
<b>Lane 5</b>	<b>30<math>\mu</math>l of ML-2 preparation</b>
<b>Lane 6</b>	<b>50<math>\mu</math>l of ML-2 preparation</b>

S-1  
S-2  
S-3

S-4  
S-5



**TABLE 3.2**                      **Protein Content of Pertussis Toxin Preparations ML-1 and ML-2 as Measured by the Method of Lowry et al., (1951).**

Toxin Sample		Protein content (mg/ml)
ML-1	1.	0.56
ML-1	2.	0.60
ML-1	3.	0.61
ML-1	4.	0.61
ML-2	1.	0.82
ML-2	2.	0.80
ML-2	3.	0.77
ML-2	4.	0.77

### 3.4.                      Isolation of pertussis toxin subunits

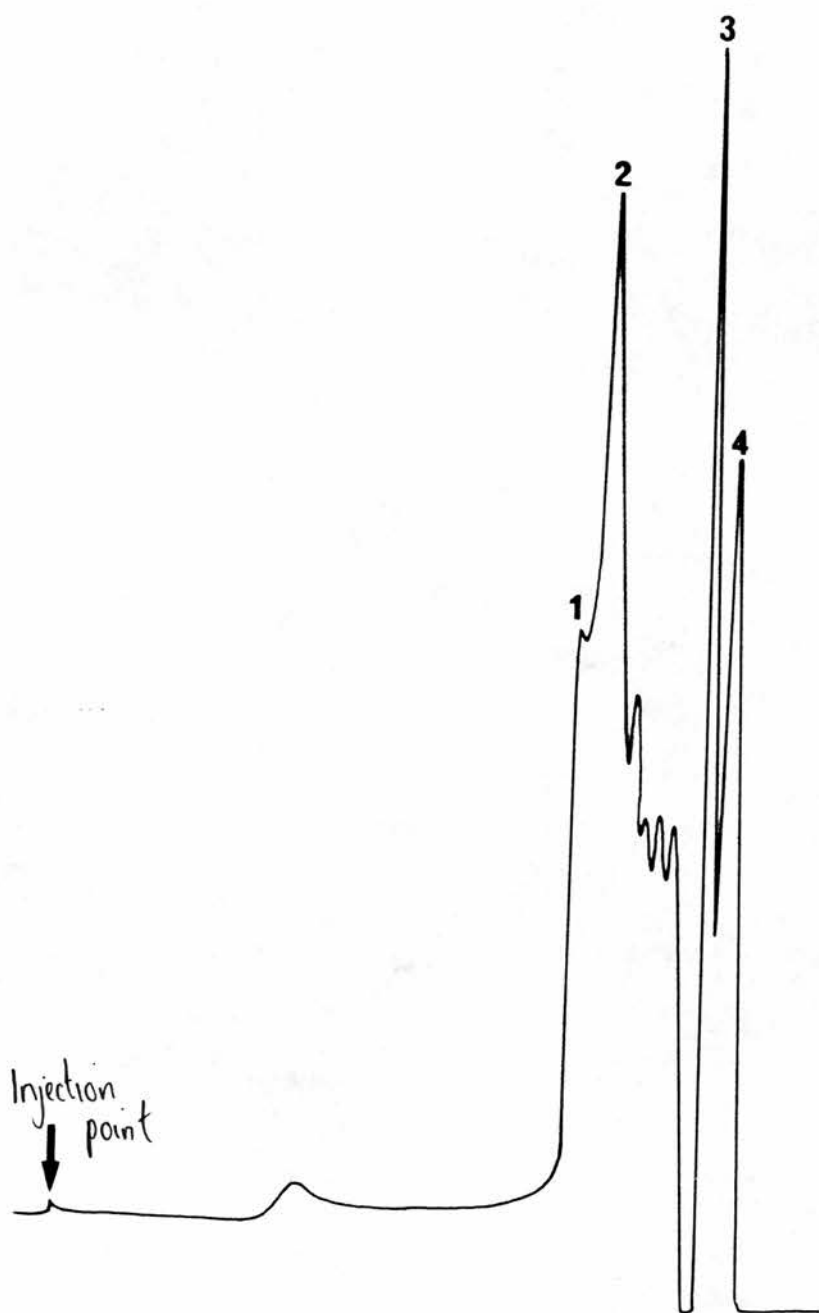
Pertussis toxin ammonium sulphate suspension was pelleted at 13 000 rpm and resuspended in 6 M guanidinium chloride, 50 mM phosphate, p.H. 6.5 buffer. This solution was *stirred overnight at room temperature* This buffer - guanidinium chloride solution was then dialysed extensively against 8 M urea with continual buffer changes to ensure that all the guanidinium chloride was removed from the sample. In 8 M urea (Rappuoli and Silvestri, 1987) pertussis toxin subunits should be dissociated into monomers of S-1, S-2, S-3, S-4 and S-5 and thus should be resolved using gel filtration FPLC.

The Superose 12 column was pre-equilibrated with 8 M urea, 50 mM potassium phosphate p.H. 6.5 buffer with five column volumes and then set at a flow rate of 0.5 ml per minute, the flow rate being kept low as the buffer is viscous and the pressure of the system must be kept below 3MPa. The toxin sample was filtered

**FIGURE 3.2**

**FPLC SEPARATION OF PERTUSSIS TOXIN SUBUNITS.  
ON THE BASIS OF MOLECULAR MASS:**

<b>1</b>	<b>-</b>	<b>S-4/S-2 DIMER</b>
<b>2</b>	<b>-</b>	<b>S-4/S-3 DIMER</b>
<b>3</b>	<b>-</b>	<b>S-1</b>
<b>4</b>	<b>-</b>	<b>S-5</b>



through a 0.22 $\mu$  filter and applied to the column. 1 ml samples were collected from the column. The trace of protein separation is shown in Figure 3.2 and those samples which contain protein, as indicated by their absorbance at 280nm, were <sup>electrophoresed</sup> on a 17.5% S.D.S. polyacrylamide gel. The gel obtained demonstrated that the amount of protein isolated was so small <sup>and so</sup> ~~prevented~~ the accurate determination of which subunits were <sup>present</sup> in each fraction.

### 3.5. DISCUSSION

In this chapter the techniques involved in the production and purification of pertussis toxin were carried out. Two toxin preparations, ML-1 and ML-2 were purified to homogeneity and an attempt at the FPLC separation of pertussis toxin subunits was undertaken.

#### 3.5.1. Purification of pertussis toxin

The toxin was purified to homogeneity by the methods of Irons and MacLennan (1979) and of Sekura et al, (1983). About 30-50 mg of toxin are generally purified from 9 litres of culture supernate by the above procedure. The purified toxin was ~~assessed~~ by protein content, SDS polyacrylamide gel electrophoresis and haemagglutination titre. Several other *in vivo* assays were also carried out on the purified toxin. These assays are only carried out on the final purified protein as they are very expensive and subject to inaccuracy making *in vitro* assays more sensible for the monitoring of fractions obtained during the toxin purification procedure. These *in vitro* assays include analysis of the NAD<sup>+</sup>-glycohydrolase activity (Moss and Vaughn, 1984; Ribeiro-Neto, et al 1985); Chinese hamster ovary cell assay (Hewlett et al, 1983; Gillenius et al, 1985) which involves the calculation of the minimum concentration of toxin required to obtain complete cell clumping and enzyme-linked immunosorbent assays (ELISA) Voller et al, 1980; Sato et al, 1983).

Toxin preparations must be very stringently tested and characterised before use

in subsequent experiments.

The toxin is stored in 50 mM tris, 0.5 M NaCl p.H. 8 buffer as a suspension in saturated ammonium sulphate. The purity of the final toxin preparation is not less than 98% which contains 0.5 - 1% lipopolysaccharide endotoxin (LPS). Pertussis toxin purified by the above procedures, as ML-1 and ML-2 is used in the production of the CAMR acellular vaccine so the purity and activity of each individual preparation must be determined to great accuracy.

### 3.5.2. Isolation of subunits

There is much controversy concerning the conditions required for the separation of pertussis toxin subunits. Tamura *et al.*, (1982) reported that the toxin dissociated into its subunits in urea concentrations greater than 2 M and that the subunits can be separated on CM-Sepharose after further exposure to 5 M urea. This separation resolved S-1, S-5 and the two dimers, and the two dimers could themselves be resolved in 8 M urea. Sekura *et al.*, (1983) reported that S-1 could be separated from the B-oligomer after exposing the toxin to 5 M urea, the S-1 being resolved on an affinity matrix which will bind the B-oligomer. Capiou *et al.*, (1986) attempted to repeat this procedure and their results suggested that the B-oligomer was not bound by the haptoglobulin - Sepharose affinity matrix and that the column eluate contained a mixture of S-1, S-3, S-4 and S-5, the column only retaining S-2 and S-4 in equal amounts. The reason for these differences are unknown.

Subunit purification was attempted in this case by exposing the toxin to 6 M guanidine followed by extensive dialysis against 8 M urea and gel filtration FPLC on a Superose 12 column. This was partially successful as it appears that the toxin has been resolved into dimers S-2 and S-4, and S-3 and S-4 and subunits S-1 and S-5 on the basis of their molecular mass and by comparison with the masses of a set of standard proteins. The yield of protein from the column however was very low and not sufficiently high to do any subsequent work with. This very low yield is probably due to the reported strong adsorption of pertussis toxin to many of the commonly used chromatography media.



## CHAPTER 4

### KINETIC ANALYSIS OF PERTUSSIS TOXIN

#### 4.1.1. INTRODUCTION

As outlined in the introduction pertussis toxin catalyses the ADP-ribosylation of Gi, the inhibitory regulatory protein of the adenylate cyclase complex (Ui et al., 1985; Ui et al., 1986). The overall effect of this ADP-ribosylation is to prevent the inhibitory action of certain hormones upon this complex, which results in an activation of the catalytic unit with unregulated amounts of cAMP being produced. Pertussis toxin catalyses the ADP-ribosylation of several other G-proteins, again causing these proteins to lose their transducing activities (Gilman, 1987; Milligan, 1988).

Gi has been purified to homogeneity from several cell types (Bokoch, et al., 1983; Katada et al., 1983; Katada et al., 1985). Purification of this protein is complex as it makes up only one part in  $10^5$  of total cellular protein and is also very labile (Gilman, 1984). As is the case with cholera toxin, additional cofactors may be necessary to demonstrate pertussis toxin catalysed ADP-ribosylation of purified Gi (Enomoto and Gill, 1979; Khan and Gilman, 1984). A simple system would therefore be much more favourable for the kinetic analysis of pertussis toxin. Tamura et al., (1982); Katada et al., (1983) and Moss et al., (1983) demonstrated that pertussis toxin had an  $\text{NAD}^+$ -glycohydrolase activity, which is not expressed by the toxin in the form by which it is secreted by *B. pertussis*, but which can be shown by incubating the toxin with reducing agents such as dithiothreitol. The  $\text{NAD}^+$ -glycohydrolase activity is the pertussis toxin catalysed hydrolysis of  $\text{NAD}^+$  to ADP-ribose and nicotinamide with water acting as the acceptor of the ADP-ribose moiety. This activity of the toxin is much simpler to study at the kinetic level than ADP-ribosylation of GTP-binding proteins.

The S-1 subunit of pertussis toxin has been shown to be activated in a number

of ways (Lim *et al.*, 1985; Moss *et al.*, 1986). The most important of these activating reagents are the sulphydryl reducing agents. The incorporation of dithiothreitol within the assay cocktail (at concentrations up to 250 mM), has been shown to increase nicotinamide release from  $\text{NAD}^+$ . This level of thiol incorporation is the universally accepted concentration of reducing reagent and results in maximum nicotinamide production from the pertussis toxin catalysed reaction. This concentration appears high if it is only required within the assay to maintain enzyme activity, strongly suggesting that it may have another function within the reaction. Moss and Vaughn (1984) demonstrated that activation would result from incubating the toxin with 20 mM dithiothreitol, again suggesting that the vast excess of reducing reagent may play an alternative role within the enzyme catalysed process. ATP and the detergent CHAPS promote the activation of pertussis toxin by DTT (Kaslow *et al.*, 1987). Burns and Manclark (1986), demonstrated that the combination of ATP and CHAPS causes the release of S-1 from the B-oligomer. These observations suggested that the activation of pertussis toxin by ATP, CHAPS and DTT may expose the key amino acids of the toxin essential in the catalytic mechanism. It has also been suggested by Moss *et al.*, (1986) that membrane phospholipids act synergistically with ATP in the activation of pertussis toxin.

In order to determine the sites of action of ATP and CHAPS, the S-1 subunit was separated from the B-oligomer by chromatographic methods and the effects of the two reagents on the isolated active subunit investigated. Only CHAPS was shown to stimulate the  $\text{NAD}^+$ -glycohydrolase activity of the isolated S-1 subunit indicating that the detergent was directly involved in the activation of S-1. ATP was shown only to activate the holotoxin; this activation was thought to result from the binding of ATP to the B-oligomer which in some way caused the S-1 subunit to dissociate from the binding component. Diphtheria toxin has been shown to have a tightly bound nucleotide (Ap Up), which must be bound to this protein for maximal activation of enzymic activity. ATP may be acting in an analogous fashion within the pertussis toxin reaction mixture by mimicking the action of a compound similar to ApUp involved in the stimulation of the enzymic activity of pertussis toxin.

Kaslow *et al.*, (1987) identified three binding sites on the toxin for these activating substances: (i) the A (adenosine) and P (polyphosphate) sites, these two sites being involved in the binding of ATP; (ii) the L-site (or lipid site) which binds activators which are hydrophobic in nature and (iii) the S (sulphydryl) site which leads to activation of the toxin when it is reduced.

Do these activating agents mimic what is happening in infected cells? Katada *et al.*, (1983), suggested that an enzymic mechanism is essential for activation of the toxin *in vivo*, this involving either disulphide bond reduction or proteolytic cleavage. This does not appear to be the case however, since Meister and Anderson (1983), demonstrated that in the presence of ATP and CHAPS, glutathione could activate pertussis toxin within the range found in eukaryote cells. This argues that cellular ATP and membrane lipids could allow intracellular glutathione to activate pertussis toxin by a non-enzymic procedure within the host cell.

There are two cysteine residues within the S-1 subunit and these form a disulphide bond in the mature toxin (Burns, 1988). It is reduction of this bond which is essential for toxin activation. Kaslow *et al.*, (1987), suggested that one, or both of these cysteine residues may be involved directly in the enzyme mechanism of the toxin. Modification of these two cysteine residues, Cys 41 and Cys 200, resulted in loss of enzymic activity. To establish which of these cysteine residues is the more important in catalysis, Kaslow *et al.*,<sup>(1987)</sup> generated a tryptic fragment of the S-1 subunit which contained the first 187 amino acids, therefore not cysteine 200, and demonstrated that this fragment was enzymically active. This suggests that Cys 41 may be in the region of S-1 which plays a critical role in the enzymic reaction catalysed by the toxin (Locht *et al.*, 1987).

The present trend of thought is that this disulphide bond plays two roles in the activity of pertussis toxin. Firstly, that the disulphide bond maintains the S-1 subunit in the appropriate conformation enabling it to bind to the B-oligomer and secondly, that it maintains S-1 in an enzymically inactive form. Reduction of this disulphide bond therefore has two effects both of which result in increased enzymic activity. Reduction of the bond stabilising an enzymically active conformation which does not reassociate with the B-oligomer and also exposes Cys 41 and the region surrounding it, this region

being important in enzymic activity.

Burns *et al.*, (1988) demonstrated that pertussis toxin has many structural similarities to cholera toxin, which has similar enzymic activities. Regions of amino acid sequence homology have been found between these toxins (Locht and Keith, 1986; Nicosia *et al.*, 1986) suggesting that these regions may be involved in the enzymic reactions of these proteins. These regions are found between amino acids 30 and 58, including Cys 41, which is further evidence that this residue plays an important role in the  $\text{NAD}^+$ -glycohydrolase and ADP-ribosyltransferase activities of the toxin.

#### 4.1.2. Kinetic models

In order to evaluate the kinetics of an enzyme catalyzed reaction the kinetic parameters  $K_m$  (Michaelis constant) and  $k_{cat}$  (the maximum capacity of the enzyme when substrate is saturating) are calculated.

These constants give an estimate of the kinetics of that reaction under the conditions likely to be found *in vivo*. Published values for the pertussis toxin catalysed  $\text{NAD}^+$ -glycohydrolase reaction differ as a consequence of the degree of activation of the enzyme. Moss and Vaughn (1984) have demonstrated that maximum  $\text{NAD}^+$ -glycohydrolase activity is obtained by the addition of 250 mM dithiothreitol within the assay system. This concentration appears exceptionally high if it is only required to maintain the toxin within an active conformation, however this is the concentration of reducing reagent routinely incorporated within the  $\text{NAD}^+$ -glycohydrolase reaction mixture. This massive concentration suggests an alternative role for these reagents and this is investigated in this chapter.

Tait and Nassau (1984) developed a model assay system for cholera toxin, using low molecular weight arginine derivatives as substrates for its ADP-ribosyltransferase reaction. This assay is similar to that of Moss and Vaughn (1984) in that it measures toxin activity indirectly by measuring the rate of nicotinamide release from  $\text{NAD}^+$ . This assay was adapted to investigate potential substrates for pertussis toxin, both those

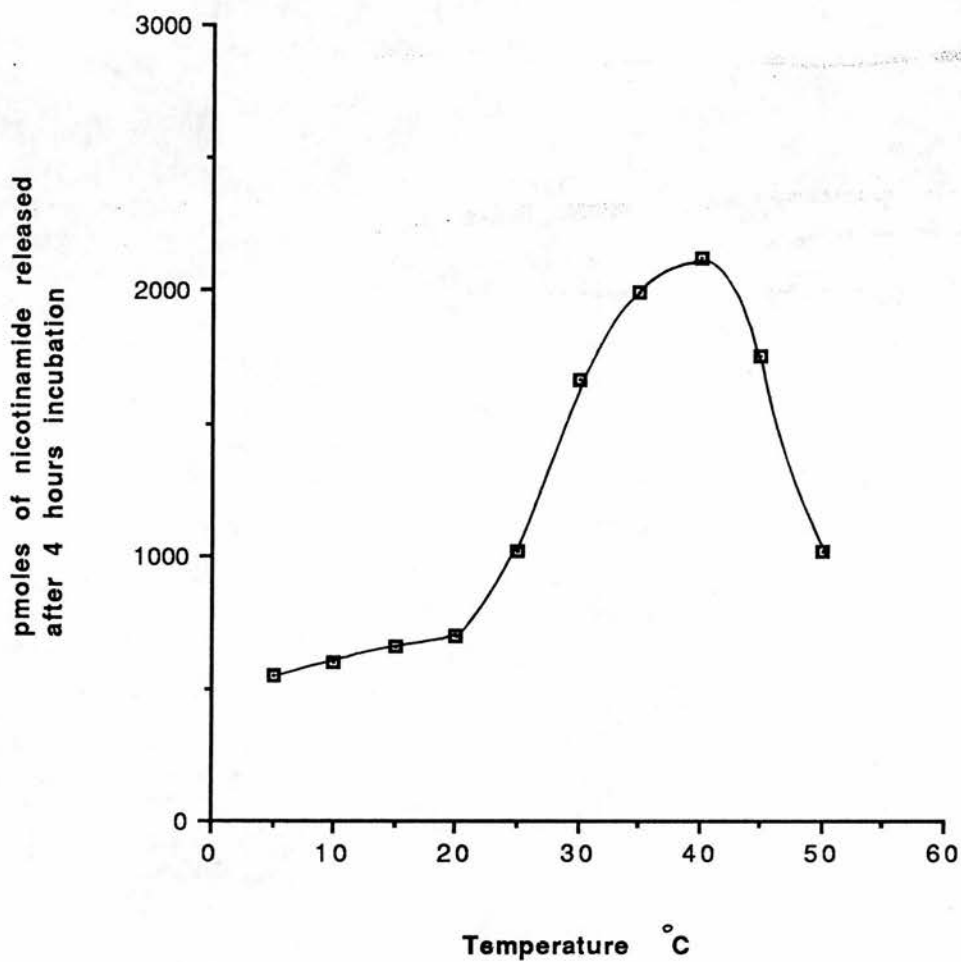
which had been shown previously to be substrates for cholera toxin and sulphydryl group containing compounds. The NAD<sup>+</sup>-glycohydrolase activity of pertussis toxin has been previously studied by Moss and Vaughn (1984); Moss *et al.*, (1986) and Kaslow *et al.*, (1987) who investigated the properties of the different activating reagents discussed in the introduction to this chapter. In the remainder of this chapter the NAD<sup>+</sup>-glycohydrolase activity of the toxin has been further investigated and the mechanism whereby several sulphydryl-reducing reagents were involved in the assay system demonstrated in an attempt to gain further information about the nature of this enzymic activity of this toxin.

## **4.2. RESULTS**

### **4.2.1. Characterisation of the NAD<sup>+</sup>-glycohydrolase assay**

Preliminary experiments were carried out to check the suitability of the assay for kinetic measurements. The NAD<sup>+</sup>-glycohydrolase activity was found to be proportional to toxin concentration over the concentration range 5-50 µg/ml. The activity was also dependent upon temperature, with activity increasing between 25°C and 40°C, above which the activity began to decrease again (Figure 4.1). NAD<sup>+</sup>-glycohydrolase experiments were routinely carried out at 30°C.

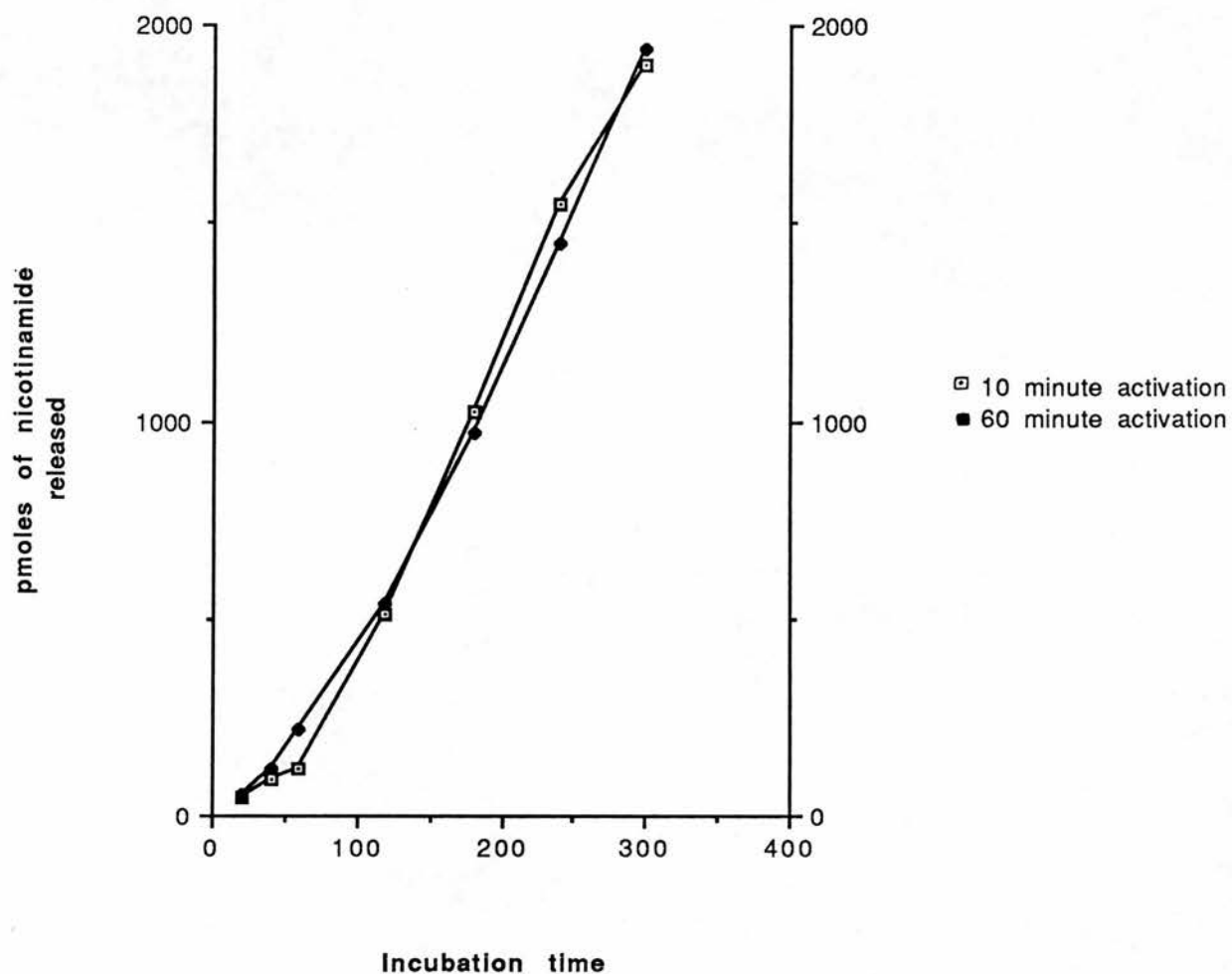
As outlined in section 2.2.3.2 the toxin must be activated via disulphide bond cleavage with reducing reagents before it will act as an NAD<sup>+</sup>-glycohydrolase. Figure 4.2 illustrates how the extent of activation affects the velocity of reaction during the initial stages of the procedure. However, after 1 hour, presumably as the toxin is in contact with 250 mM reducing agent within the assay cocktail, the toxin is fully active catalysing release of nicotinamide at the same rate independent of activation time. A pre-activation period of 30 minutes in 20 mM dithiothreitol and an assay time of 4 hours were chosen for this assay system. The linear progress curve followed after the first



**FIGURE 4.1**

The effect of temperature on the rate of pertussis toxin catalysed NAD<sup>+</sup> glycohydrolase.

Assays were routinely carried out at 30°C



**FIGURE 4.2**

The effect of pre-activation time on the rate of pertussis toxin catalysed  $\text{NAD}^+$ -glycohydrolase.

30 minute pre-activation at  $30^\circ\text{C}$  was routinely carried out.



hour of the assay, demonstrates that once fully active the conditions of the assay do not change throughout the assay period, therefore there has been no significant depletion, inactivation of enzyme, or inhibition of the enzyme with product.

#### 4.2.2. Controls of NAD<sup>+</sup>-breakdown

As the NAD<sup>+</sup>-glycohydrolase assay measures the release of nicotinamide from NAD<sup>+</sup> catalysed by pertussis toxin, it is important to include controls to take account of nicotinamide produced by the non-enzymic breakdown of NAD<sup>+</sup>. This is a greater problem when the assay is carried out in buffers of p.H values 7.0 and above, especially in the presence of phosphate ions (Colowick *et al.*, 1951). However, phosphate has been shown to promote maximum nicotinamide release from a cholera toxin catalysed NAD<sup>+</sup>-glycohydrolase system involving the same assay procedure (Moss *et al.*, 1976; Tait and Nassau, 1984). The assays were thus performed in a phosphate buffer system but at the slightly acid p.H of 6.5 to minimise the degradation of NAD<sup>+</sup>.

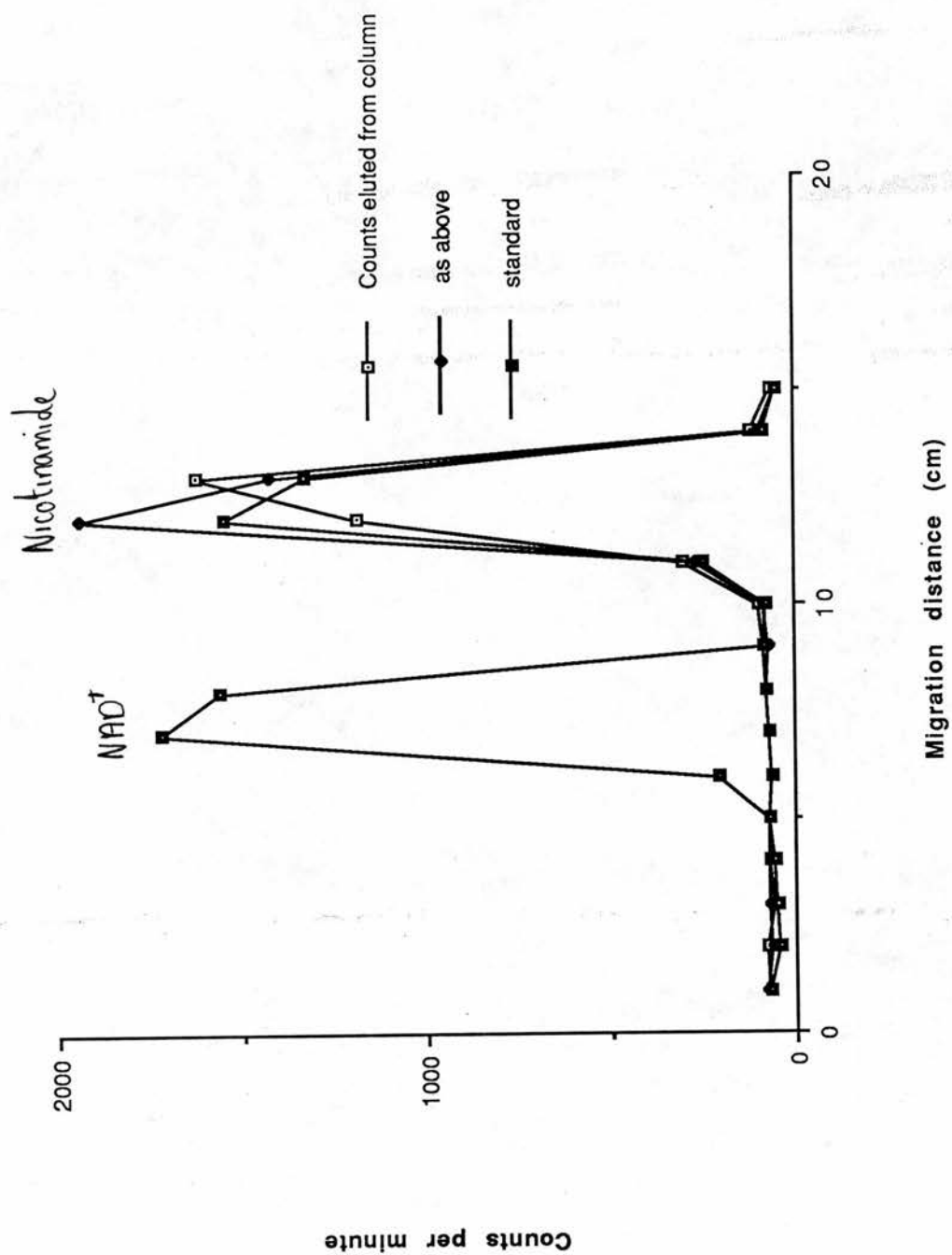
Controls were included for each assay sample, at each concentration of NAD<sup>+</sup>, to measure the non-enzymic breakdown of NAD<sup>+</sup>. These controls contained all of the assay constituents except enzyme. Non-enzymic breakdown was typically less than 5% of the total NAD<sup>+</sup> added under the conditions of the assay (4 hours at 30°C).

As a final control, paper chromatographic separation of 1μCi of both [4-<sup>3</sup>H]NAD<sup>+</sup> and [<sup>3</sup>H] nicotinamide was carried out to demonstrate the efficiency of the Dowex AG-1X resin to separate the reaction products. The experimental procedure is discussed in section 2.2.3.3. Figure 4.3 shows the results of this separation and demonstrates that of the [<sup>3</sup>H] nicotinamide added 92-98% was recovered following Dowex AG-1X chromatography and of the [4-<sup>3</sup>H] NAD<sup>+</sup>, 0.5-0.9% leached off the column.



**FIGURE 4.3**

**DEMONSTRATION OF THE EFFICIENCY OF THE DOWEX AG-1X2 RESIN FOR SEPARATING NICOTINAMIDE AND NAD<sup>+</sup>**

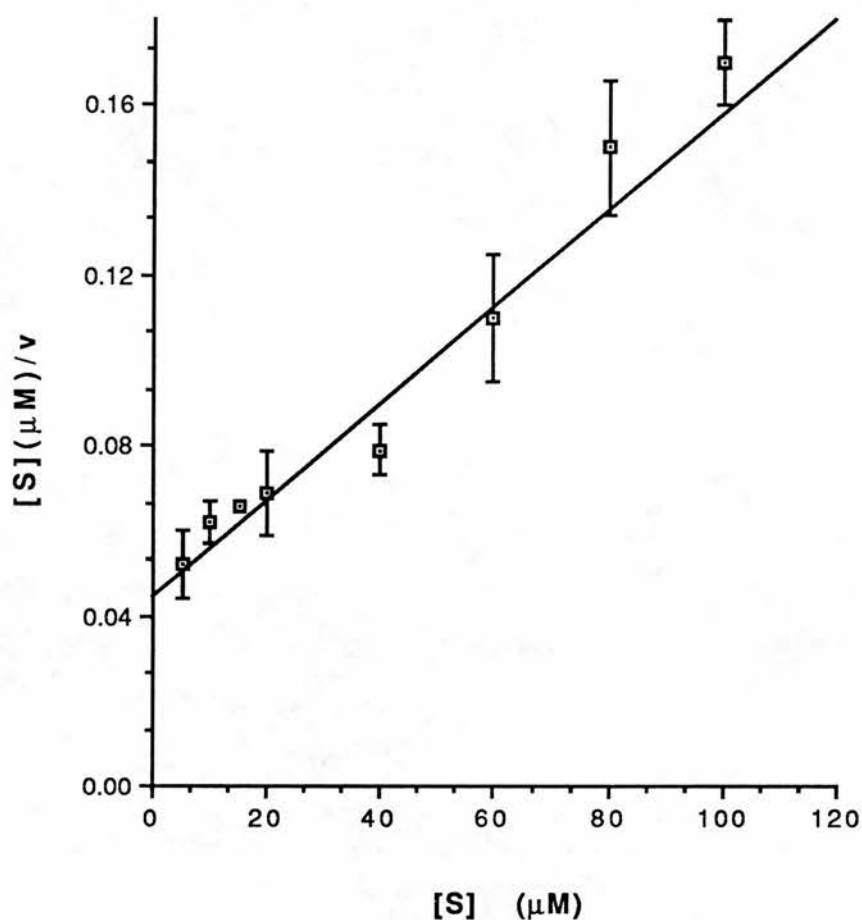


#### 4.2.3. Kinetics of the NAD<sup>+</sup>-glycohydrolase reaction

The rate of [<sup>3</sup>H] nicotinamide release from [4-<sup>3</sup>H] NAD<sup>+</sup> catalysed by pertussis toxin was measured at various concentrations of NAD<sup>+</sup>. The results are presented as Lineweaver-Burk (Lineweaver *et al.*, 1934), Hanes (Hanes 1932) and direct linear plots (Cornish-Bowden and Eisenthal, 1978), Figures 4.4, 4.5 and 4.6. The K<sub>m</sub> values calculated differed slightly between plots, the results are shown in the Figures. A k<sub>cat</sub> of 0.25 min<sup>-1</sup> was derived from this data. The k<sub>cat</sub> value assumes that all of the protein present is enzyme and that all enzyme is active. This is a valid assumption in this case as the protein preparation was very pure, containing only pertussis toxin as shown by SDS polyacrylamide gel electrophoresis, and under the conditions of this assay system the protein should remain active throughout its duration.

This K<sub>m</sub> value is also calculated at a dithiothreitol concentration of 250 mM which has been shown to be required for maximum pertussis toxin NAD<sup>+</sup>-glycohydrolase activity (Moss and Vaughn, 1984). Experiments had previously demonstrated that as more thiol was added to the system, up to these excessively high concentrations, more nicotinamide was released and 250 mM dithiothreitol became an accepted constituent of the assay mix.

Moss *et al.*, (1983) investigated the change in rate of nicotinamide release via pertussis toxin catalysed NAD<sup>+</sup> in the presence of a number of reducing reagents and these results are shown in Table 4.1. The results demonstrate that mercaptoethanol, dithiothreitol, glutathione and cysteine can all activate pertussis toxin and that increasing the concentration of any of these reducing reagents, results in increased nicotinamide release. However, cholera toxin catalysed NAD<sup>+</sup>-glycohydrolase, which involves a similar activation procedure, requires only 5 mM of reducing agent (Galloway and van Heyningen, 1986), this concentration of reducing agent is much more typical of that required for reduction of a disulphide bond. An investigation into the effect of the



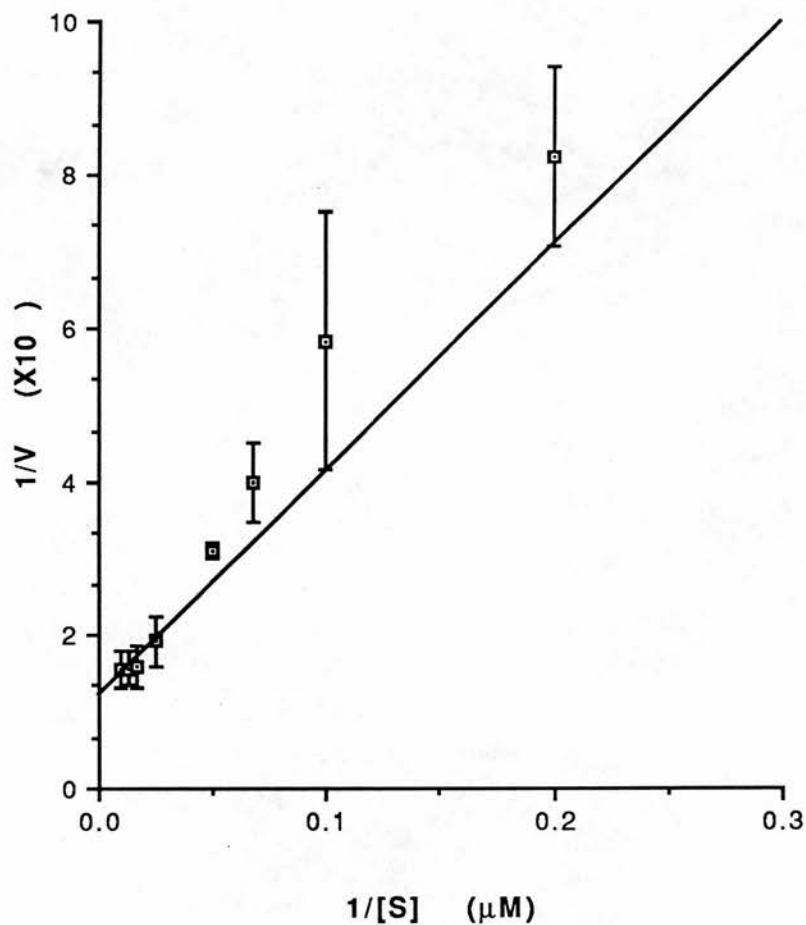
**FIGURE 4.4**

**HANES PLOT OF PERTUSSIS TOXIN CATALYSED  $\text{NAD}^+$ -GLYCOHYDROLASE.**

$$K_m = 30 \pm 5 \mu\text{M}$$

$$[S]/v = \mu\text{M NAD}^+ / \text{pmoles of nicotinamide released per hour}$$

$$[S] = \text{NAD}^+ \text{ concentration } (\mu\text{M})$$



**FIGURE 4.5**

**LINEWEAVER-BURK PLOT OF PERTUSSIS TOXIN  
CATALYSED  $\text{NAD}^+$ -GLYCOHYDROLASE.**

$$K_m = 27 \pm 3 \mu\text{M}$$

$$1/V = 1/\text{pmoles of nicotinamide released per hour}$$

$$1/[S] = 1/\text{NAD}^+ \text{ concentration } (\mu\text{M})$$

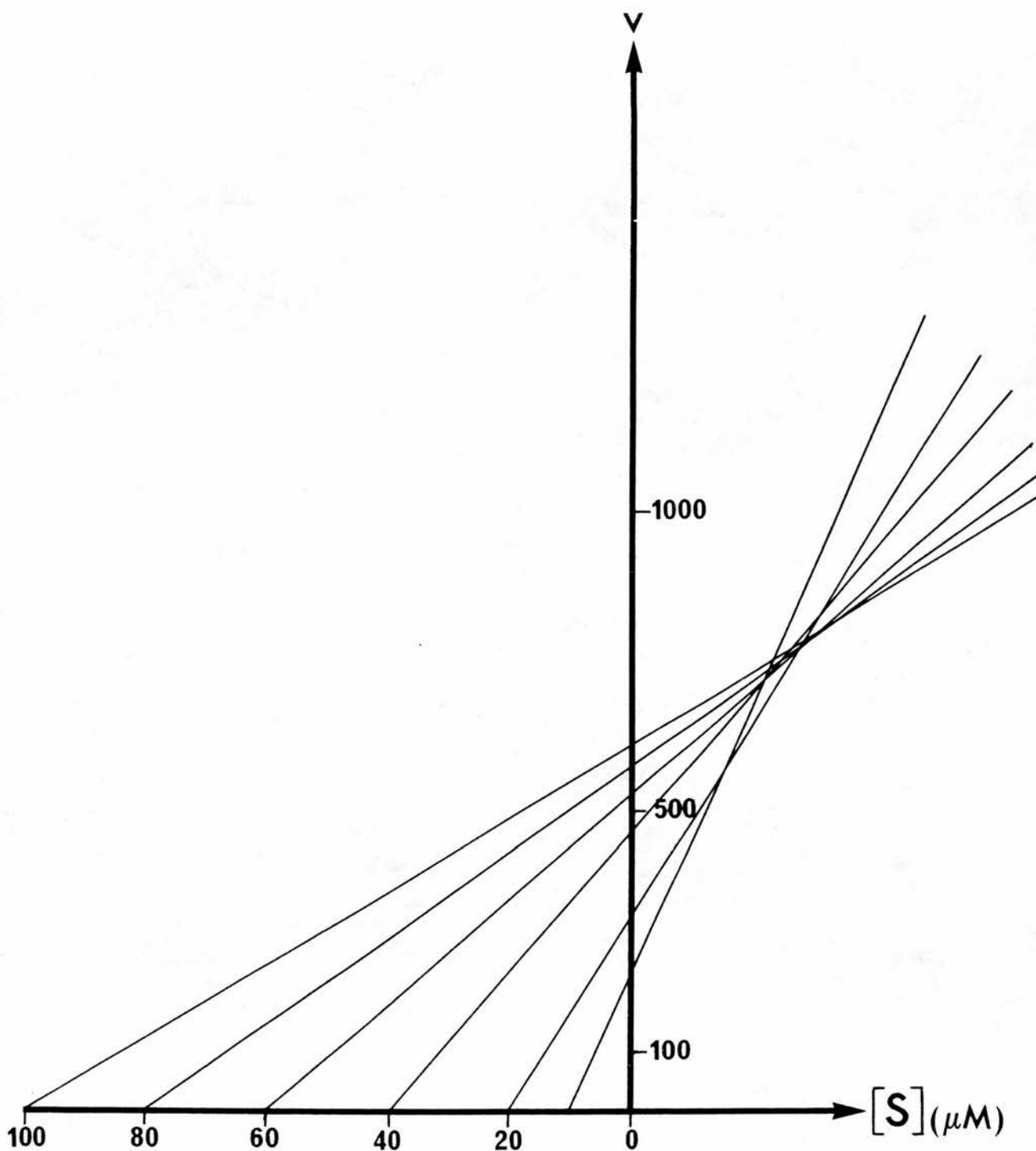


FIGURE 4.6

EISENTHAL CORNISH-BOWDEN PLOT FOR PERTUSSIS  
TOXIN CATALYSED  $NAD^+$ -GLYCOHYDROLASE.

$K_m$  for  $NAD^+$  =  $28 \mu M$

$V$  = pmols of nicotinamide released per hour  
 $[S]$  =  $NAD^+$  concentration

sulphydryl group was next carried out in order to determine whether these reagents played any other role within the  $\text{NAD}^+$ -glycohydrolase reaction mechanism apart from activation.

**Table 4.1** Effects of increasing thiol concentrations on release of nicotinamide by pertussis toxin catalysed  $\text{NAD}^+$ -glycohydrolase. (Moss *et al.*, 1983)

Additions	Nicotinamide released (pmoles)
None	0
Dithiothreitol 20mM	1140
250mM	5870
Glutathione 20mM	240
250mM	393
Cysteine 20mM	359
250mM	649
Mercaptoethanol 20mM	401
250mM	4375

#### 4.2.4. Kinetics of $\text{NAD}^+$ -glycohydrolase reaction with sulphydryl group as substrate

The amino acid which accepts the ADP-ribose moiety from the pertussis toxin catalysed ADP-ribosylation reaction has been suggested to be either aspartate (Hurley *et al.*, 1984) asparagine (Abood *et al.*, 1982; Manning *et al.*, 1984) or cysteine (West *et al.*,

1985). It is now thought most likely to be cysteine. West *et al.*, (1985) demonstrated that a cysteine residue in transducin is the amino acid which accepts ADP-ribose. This group sequenced the tryptic peptide of transducin which was ADP-ribosylated by pertussis toxin and HPLC analysis identified cysteine as the target amino acid. This being the case the sulphydryl group may be acting as a substrate by accepting the ADP-ribose, produced by the pertussis toxin catalysed  $\text{NAD}^+$ -glycohydrolase reaction.

To investigate this possibility some basic enzyme kinetics were initially carried out with the sulphydryl group as the variable. The initial velocity of [ $^3\text{H}$ ] nicotinamide release from  $\text{NAD}^+$  was measured at a saturating  $\text{NAD}^+$  concentration of 100  $\mu\text{M}$  and at various concentrations of sulphydryl reagent. The reagents used were dithiothreitol, cysteine, mercaptoethanol and glutathione. The results are shown as Hanes and direct linear plots in Figures 4.7 - 4.14. In all cases increasing concentrations of thiol increased the rate of reaction until saturation was achieved. From conventional enzyme kinetic analysis of the effects of these sulphydryl reagents at fixed  $\text{NAD}^+$  concentration,  $K_m$  values for the sulphydryl groups were calculated. The estimated  $K_m$  values from Hanes plots were 105 mM for cysteine, 127 mM for dithiothreitol, 110 mM for mercaptoethanol and 102 mM for glutathione. This indicates that the sulphydryl reagents are acting as substrates for the reaction.

#### 4.2.5. Isolation of an ADP-ribose-sulphydryl compound

The ADP-ribosylation reaction was carried out as previously described with 100  $\mu\text{M}$   $\text{NAD}^+$  and 250 mM cysteine in the final reaction mixture. The reaction products were filtered to remove all traces of protein and loaded onto an HPLC system. The column used was a reverse-phase ODS-HYP 2734 column (25cm by 4.6mm), the mobile phase was 90% (v/v) 10 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ , 10% (v/v) methanol, p.H 3.5 which was run at a flow rate of 1 ml/min, using Altex equipment. The absorbance of the eluate was monitored at 254 nm.

Figure 4.15 shows the separation by HPLC of standard solutions of ADP-



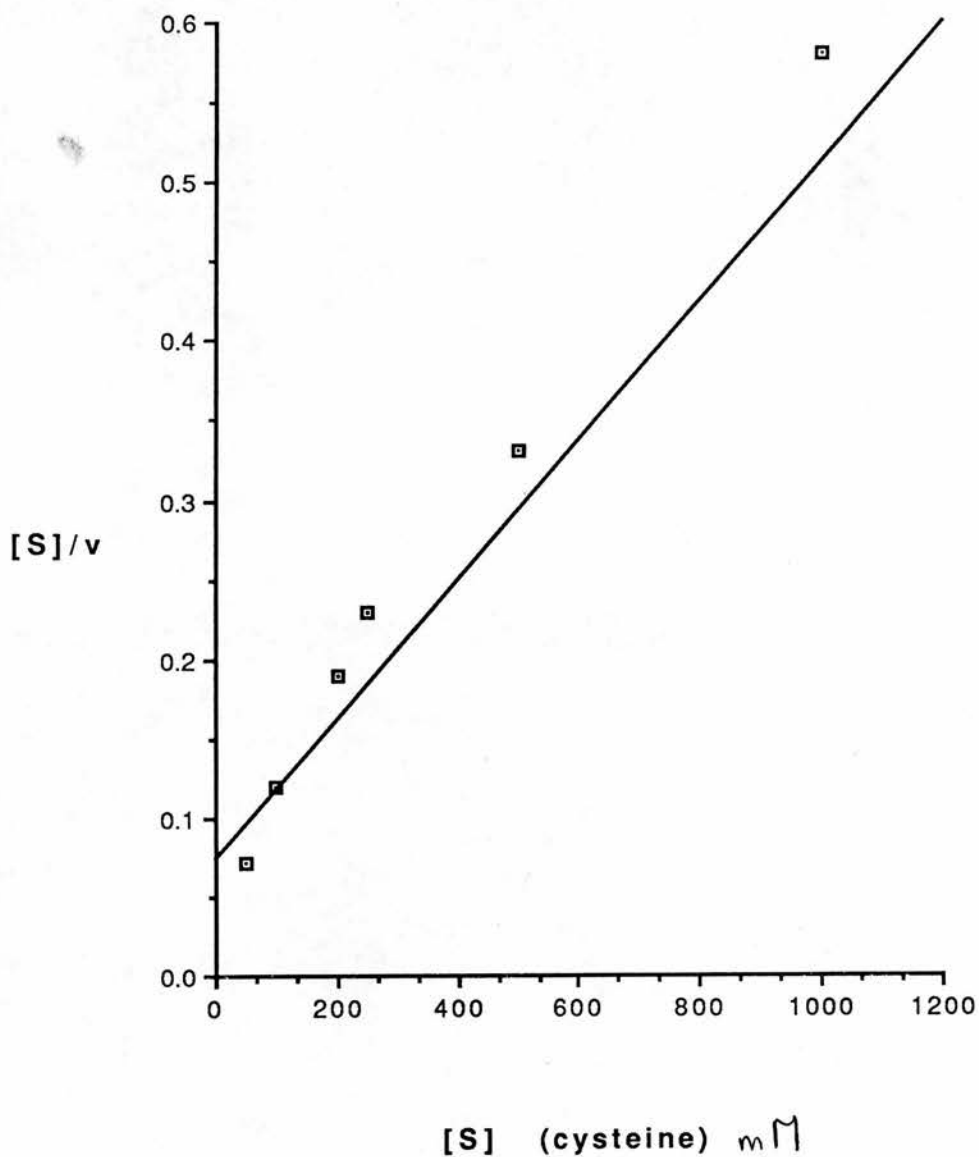


FIGURE 4.7

HANES PLOT OF PERTUSSIS TOXIN CATALYSED NAD<sup>+</sup>-  
GLYCOHYDROLASE WITH CYSTEINE AS SUBSTRATE.

$$K_m = 105 \text{ mM}$$

$$[S]/v = \text{Cysteine concentration} / \text{pmoles of nicotinamide released per hour}$$

$$[S] = \text{mM cysteine}$$

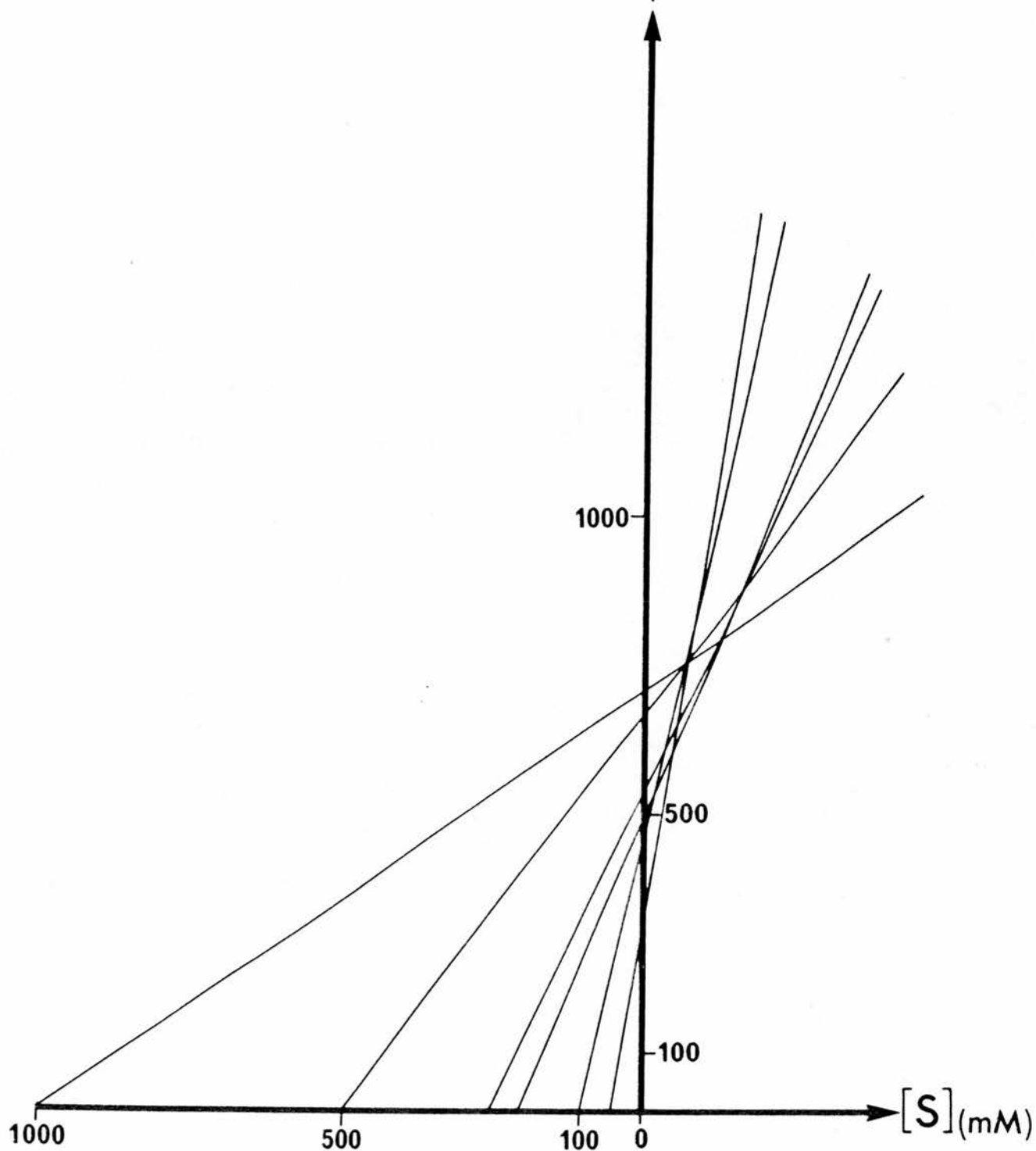


FIGURE 4.8

EISENTHAL CORNISH-BOWDEN PLOT FOR PERTUSSIS  
TOXIN CATALYSED  $\text{NAD}^+$ -GLYCOHYDROLASE WITH  
CYSTEINE AS SUBSTRATE.

$K_m = 110\text{mM}$

$v =$  pmols of nicotinamide released per hour

$[S] =$  cysteine concentration

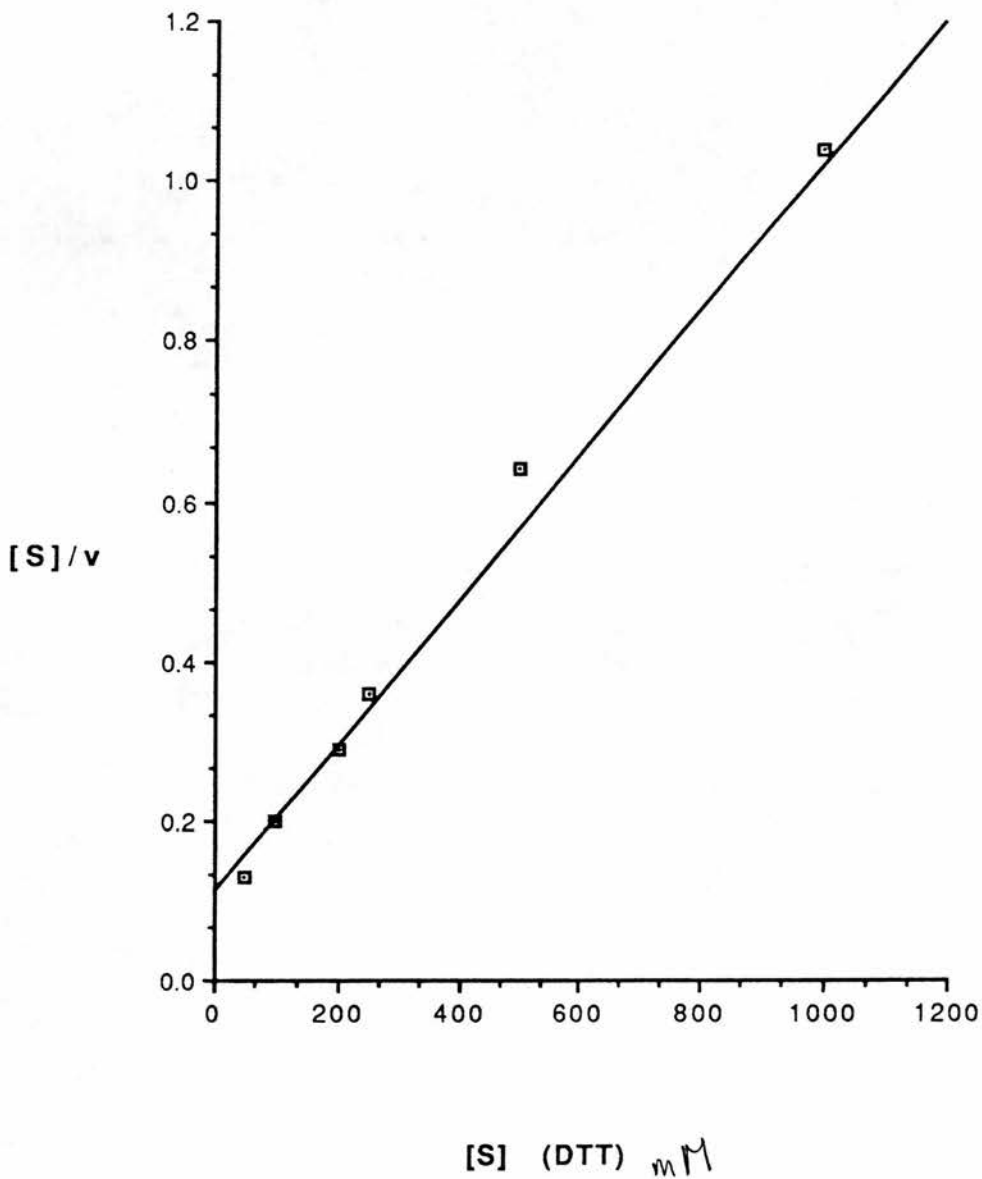


FIGURE 4.9

HANES PLOT OF PERTUSSIS TOXIN CATALYSED  $\text{NAD}^+$ -GLYCOHYDROLASE WITH DTT AS SUBSTRATE.

$$K_m = 127 \text{ mM}$$

$[S]/v$  = dithiothreitol concentration /  $\mu$ moles of nicotinamide released per hour

$[S]$  = mM dithiothreitol

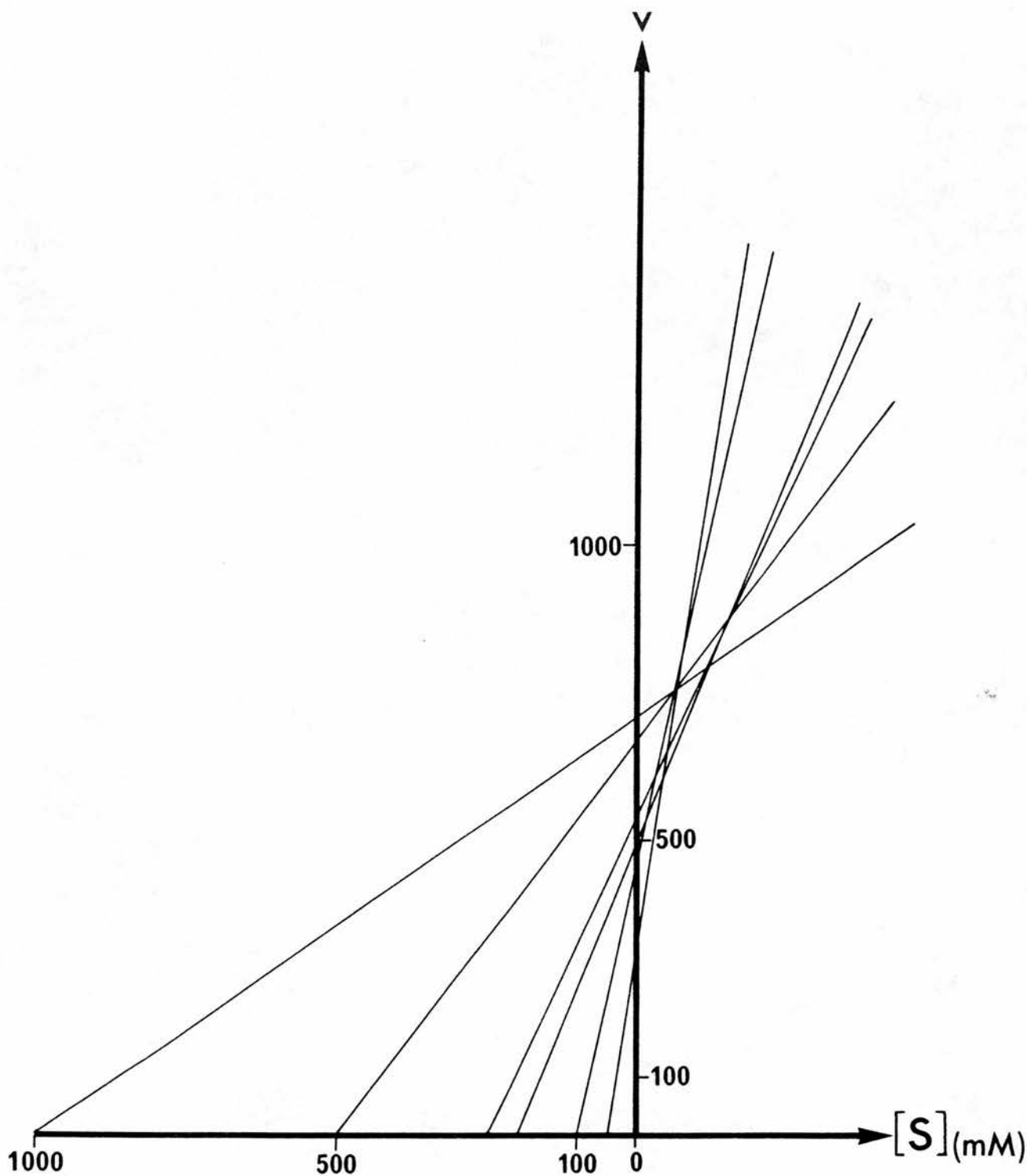


FIGURE 4.10

EISENTHAL CORNISH-BOWDEN PLOT FOR PERTUSSIS  
TOXIN  $\text{NAD}^+$ -GLYCOHYDROLASE WITH DITHIOTHREITOL  
AS SUBSTRATE.

$K_m = 110\text{mM}$

$[S] = \text{mM dithiothreitol}$

$v = \text{pmoles of nicotinamide released per hour}$

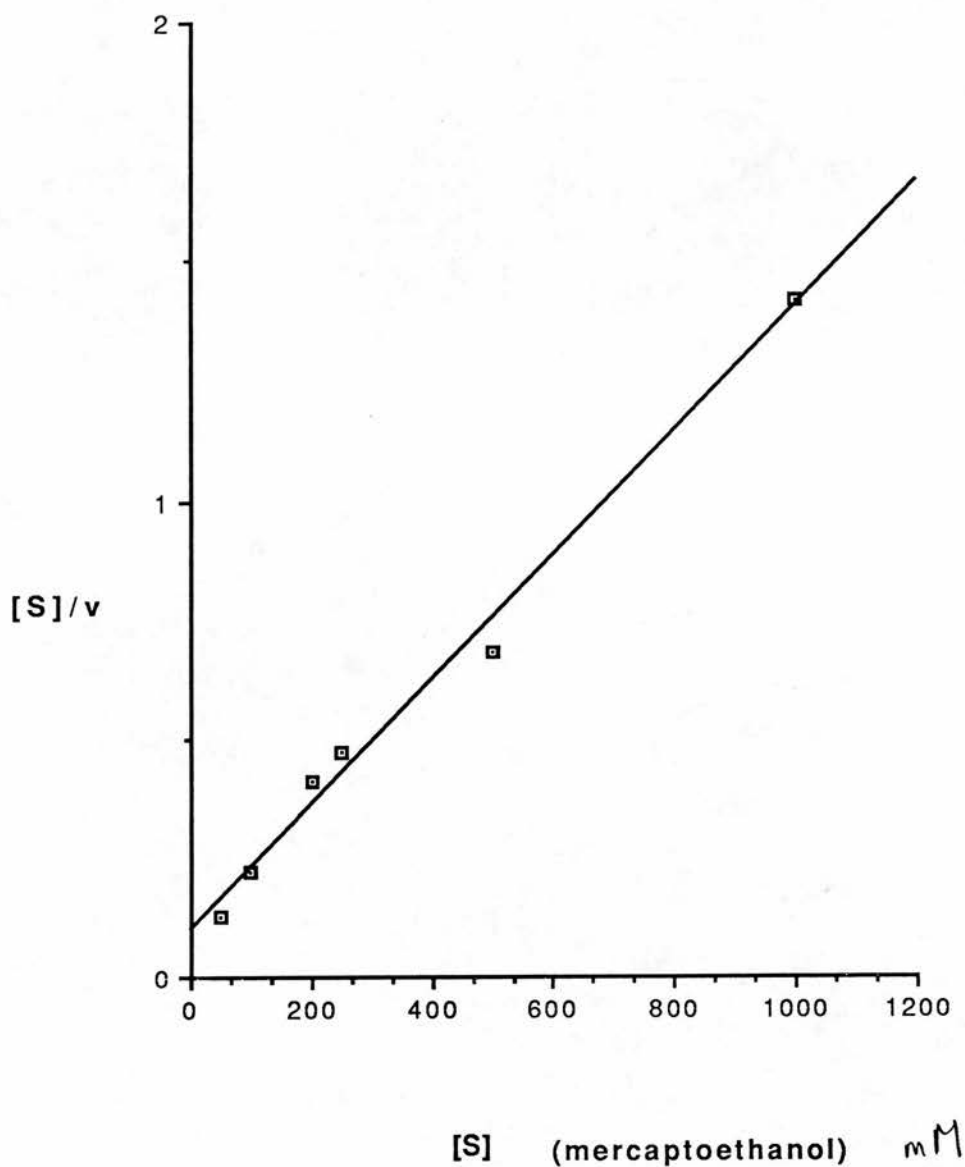


FIGURE 4.11

HANES PLOT OF PERTUSSIS TOXIN CATALYSED  $\text{NAD}^+$ -  
GLYCOHYDROLASE WITH MERCAPTOETHANOL AS  
SUBSTRATE.

$$K_m = 110 \text{ mM}$$

$$[S]/v = \text{mercaptoethanol concentration} / (\text{pmoles of nicotinamide released per hour})$$

$$[S] = \text{mercaptoethanol concentration}$$

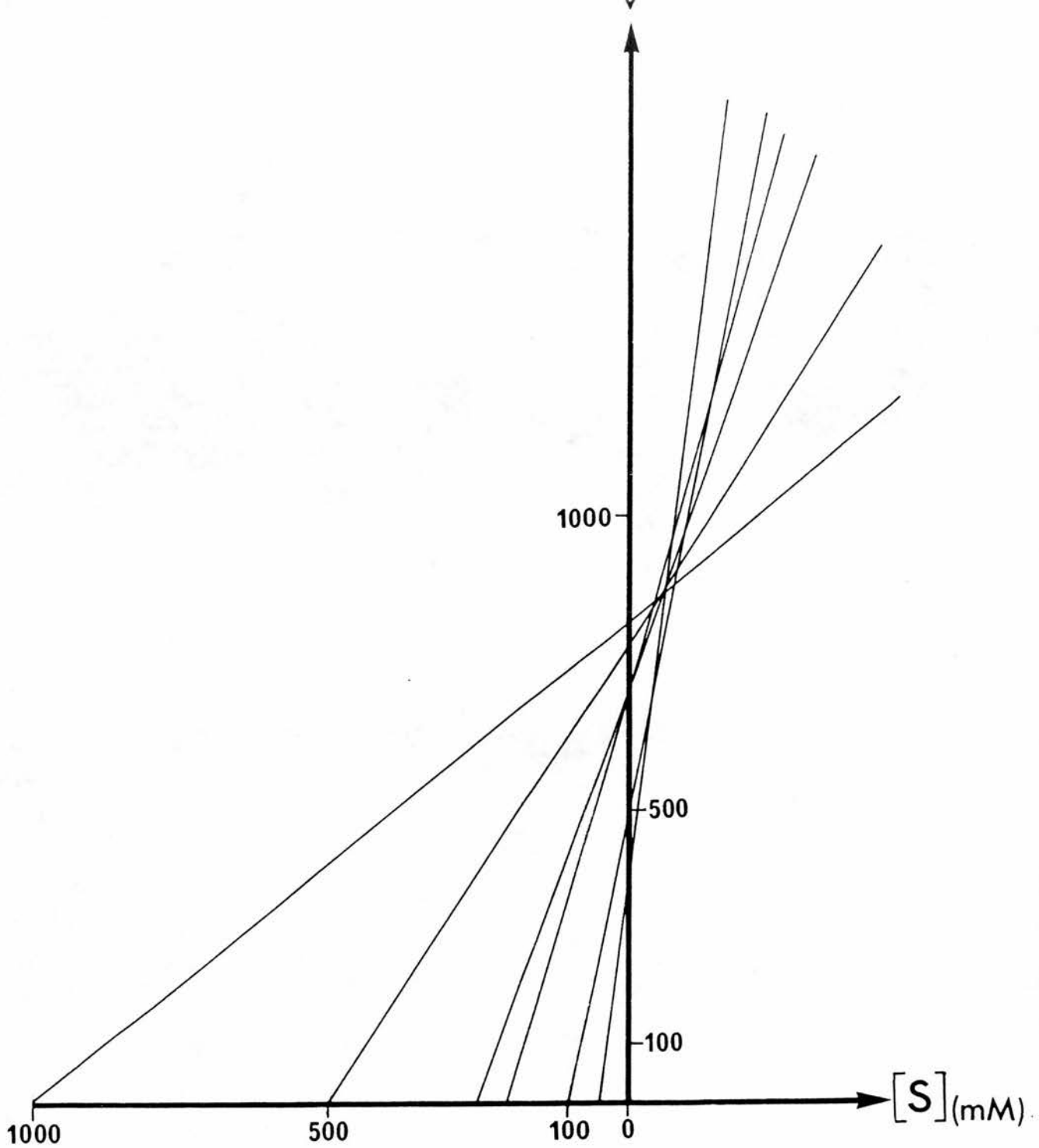


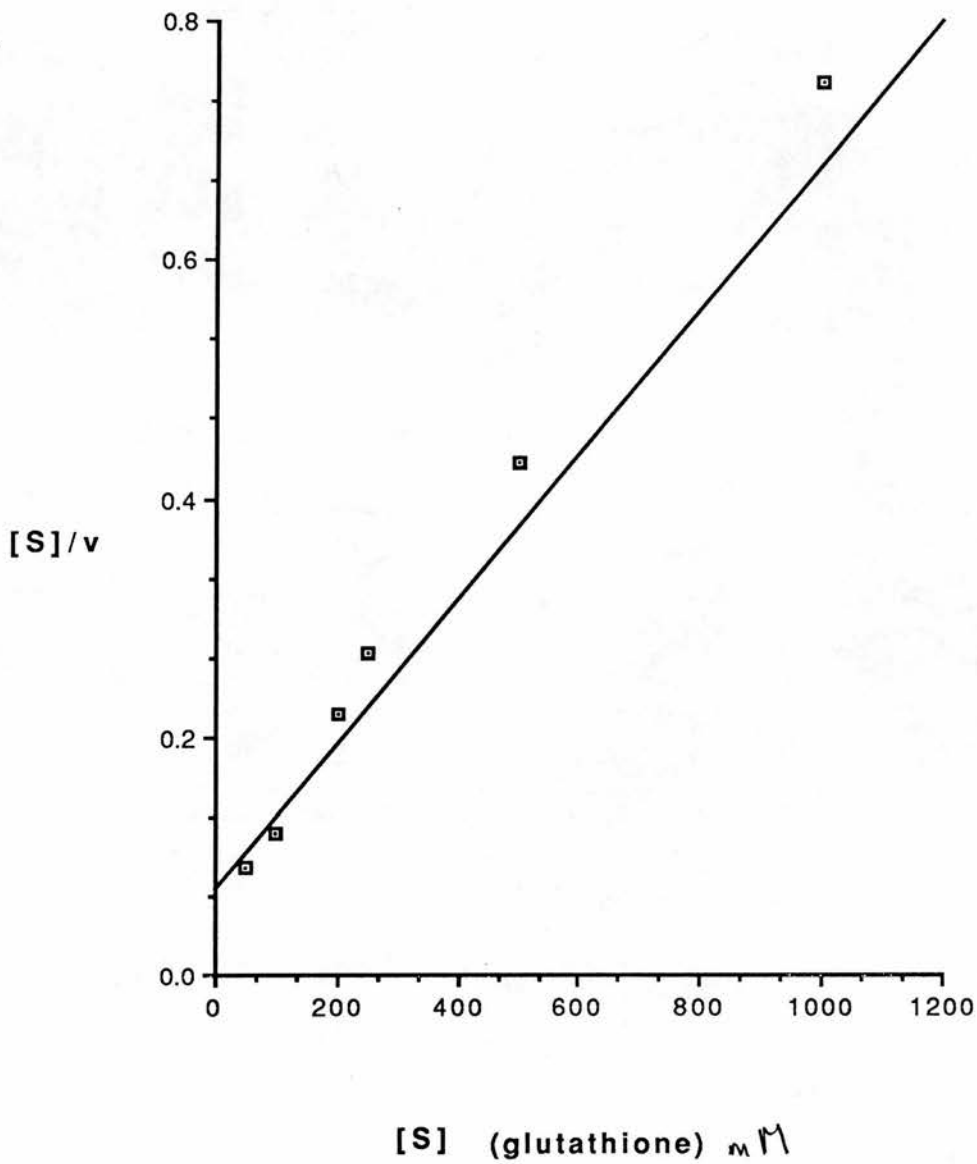
FIGURE 4.12

EISENTHAL CORNISH-BOWDEN PLOT FOR PERTUSSIS  
TOXIN CATALYSED  $\text{NAD}^+$ -GLYCOHYDROLASE WITH  
MERCAPTOETHANOL AS SUBSTRATE.

$K_m = 90\text{mM}$

$v =$  pmols of nicotinamide released per hour

$[S] =$  mM mercaptoethanol



**FIGURE 4.13**

**HANES PLOT OF PERTUSSIS TOXIN CATALYSED NAD<sup>+</sup>-GLYCOHYDROLASE WITH GLUTATHIONE AS SUBSTRATE.**

**Km = 102mM**

$[S]/v$  = glutathione concentration / pmols of nicotinamide released per hour

$[S]$  = mM glutathione

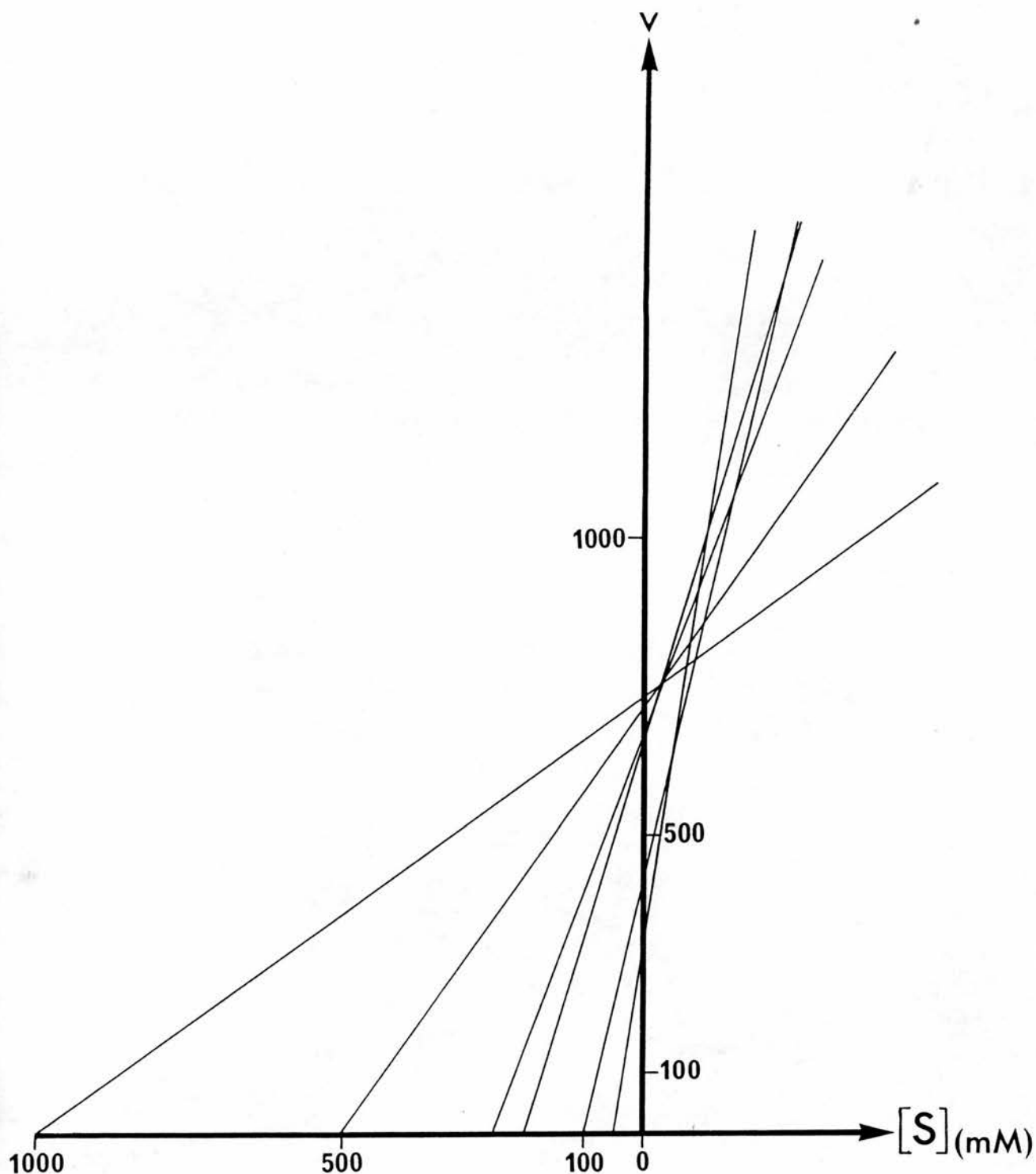


FIGURE 4.14

EISENTHAL CORNISH-BOWDEN PLOT FOR PERTUSSIS  
TOXIN CATALYSED  $NAD^+$ -GLYCOHYDROLASE WITH  
GLUTATHIONE AS SUBSTRATE.

$K_m = 102mM$

$V =$  pmoles of nicotinamide released per hour

$[S] =$  mM glutathione



ribose,  $\text{NAD}^+$ , AMP and nicotinamide. Nicotinamide absorbs very weakly at 254 nm, so the concentration of the standard nicotinamide solution was made 10 times greater than that of the other compounds. The products of the above enzyme catalysed reaction ( $100\ \mu\text{M}\ \text{NAD}^+$  in the presence of 250 mM cysteine for 4 hours at  $30^\circ\text{C}$ ) were applied to the column and ADP-ribose and  $\text{NAD}^+$  could be identified from the absorbance trace of the eluate. Figure 4.16 shows the HPLC trace obtained from the reaction products. As mentioned earlier in this section, nicotinamide absorbs very weakly at 254nm and is also much retarded in this system. This makes nicotinamide very difficult to detect using this HPLC system and, in fact impossible at the very low concentrations produced by the pertussis toxin catalysed reaction. Cysteine does not absorb at 254 nm and cannot be detected from the trace. The final reaction product cannot be identified by comparison with the standard trace. This reaction product may be the ADP-ribosyl cysteine suggested from the kinetic data as its elution position is compatible with that assumption. When isolating the ADP-ribosyl-cysteine produced from the pertussis toxin catalysed ADP-ribosylation of transducin, West *et al.*, (1985) showed that this product was eluted between ADP-ribose and  $\text{NAD}^+$  when using a very similar HPLC system.

To confirm the identities of the reaction products, radioactively labelled compounds [adenine-2,8- $^3\text{H}$ ]  $\text{NAD}^+$  ( $2.5\ \mu\text{Ci}\ \text{mmol}^{-1}$  in the mixture) and [ $^{14}\text{C}(\text{U})$ ] cysteine ( $1.3\ \mu\text{Ci}\text{-mmol}^{-1}$ ) were incorporated into the reaction mixture. The ADP-ribosylation reaction procedure was repeated as above and the filtered solution applied to the HPLC column. Eighty x 5 drop fractions (total volume about 0.5 ml) were collected from the column and each fraction assayed for both  $^3\text{H}$  and  $^{14}\text{C}$  radioactivities. Figure 4.17 shows the results. By comparing the radioactivity in each fraction with the HPLC trace of the reaction products it is clear that the unknown reaction product contains both  $^{14}\text{C}$  and  $^3\text{H}$ , and so is compound containing both cysteine and the adenine moiety of  $\text{NAD}^+$ . This peak was collected from many HPLC runs, freeze dried to concentrate the compound, resuspended in the HPLC buffer and Figure 4.18a shows

an HPLC run of the isolated assay product.

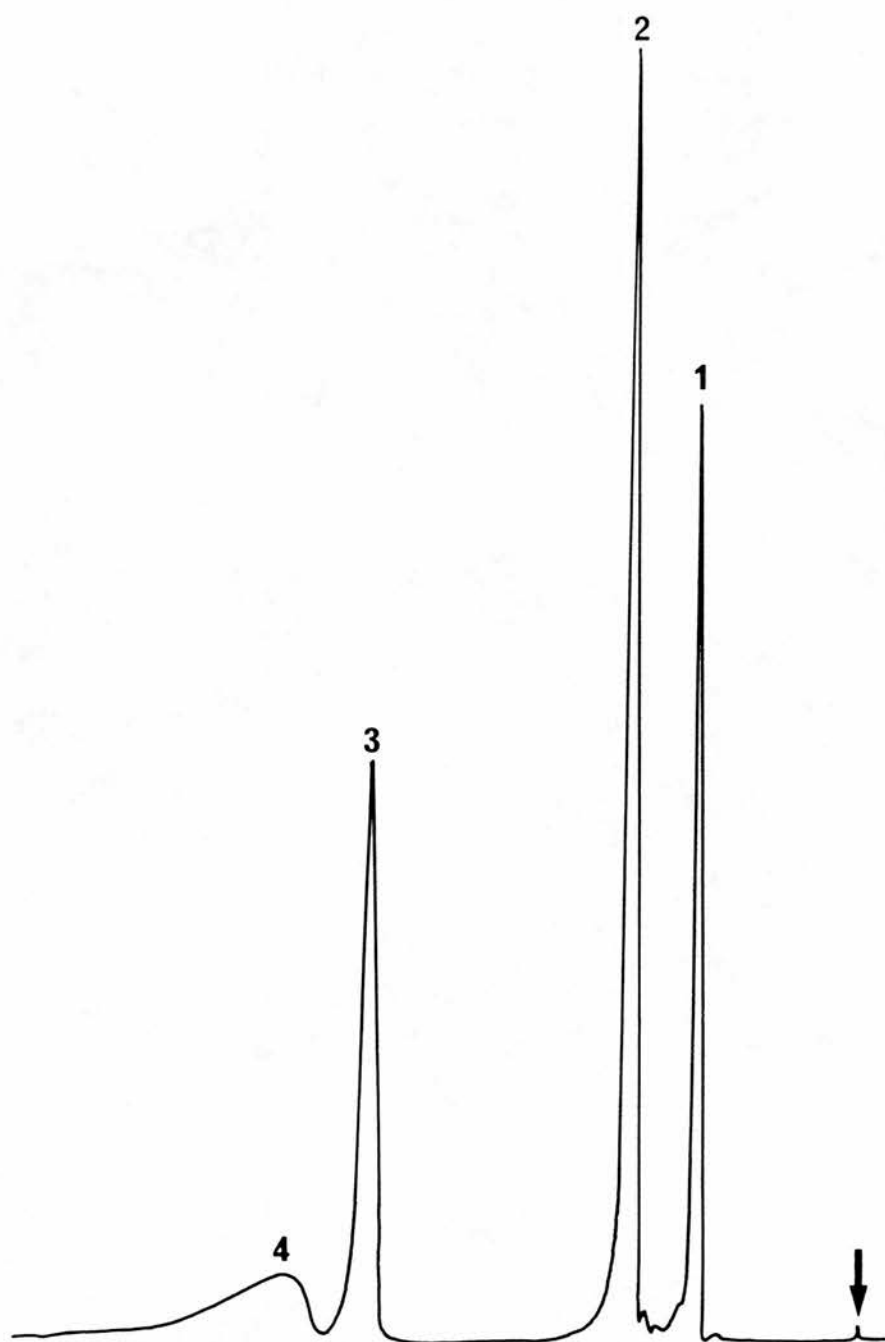
#### 4.2.6. Treatment with phosphodiesterase

If the unknown compound is ADP-ribosyl-cysteine, then treatment with snake venom phosphodiesterase should release AMP and phosphoribosyl cysteine as reaction products. A sample of the purified material (as shown in Figure 4.18a) was incubated with a phosphodiesterase from *Crotalus durissus terifficans* (20 $\mu$ g/ml) in 50 mM phosphate buffer p.H 7.5, for 5 hours at 37°C. The products of the phosphodiesterase reaction were filtered and applied to the HPLC column. Figure 4.18b shows the trace obtained demonstrating that AMP has been released by the action of phosphodiesterase, Figure 4.18c showing the proposed enzymic reaction. The other reaction product identified from this trace was a small amount of the starting material. Phosphoribosyl-cysteine does not absorb at 254 nm so its elution position could not be determined from the HPLC trace.

The isolated ADP-ribosyl-cysteine reaction product is radioactive, being labelled with both  $^{14}\text{C}$  on the cysteine moiety and  $^3\text{H}$  on the adenine constituent of this product. In an attempt to locate phosphoribosyl cysteine the phosphodiesterase reaction was repeated and 90 x 10 drop fractions were collected from the column, directly into scintillant, and each fraction counted for both  $^{14}\text{C}$  and  $^3\text{H}$  radioactivities. As the total amount of radioactivity applied to the column was small, the differences between each fraction were not great, but sufficient to identify several peaks of radioactivity. The results are shown in Figure 4.19. The peaks of radioactivity identified were:

- (i)  $^3\text{H}$  and  $^{14}\text{C}$  peak corresponding to the starting material
- (ii)  $^3\text{H}$  peak corresponding to AMP
- (iii)  $^{14}\text{C}$  peak

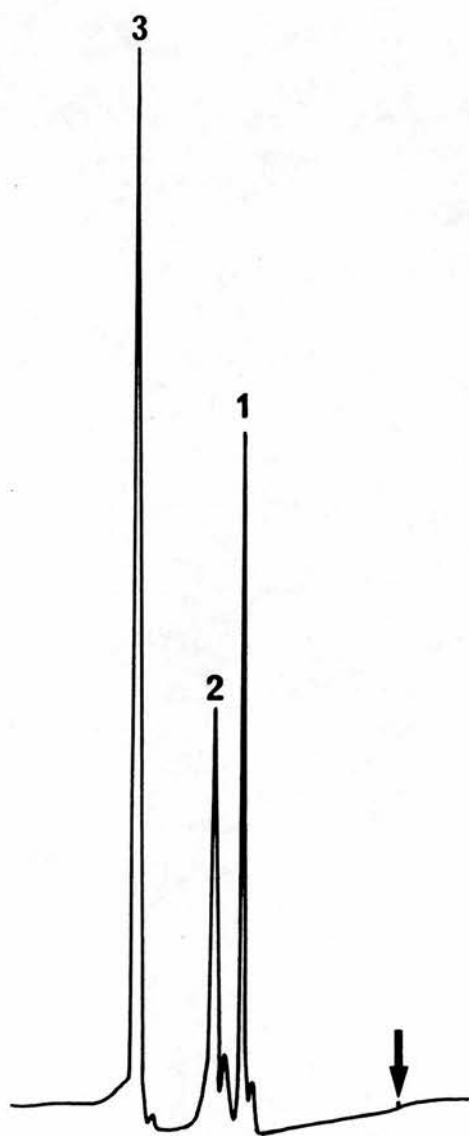
The final  $^{14}\text{C}$  peak was eluted at a position compatible with it being phospho-ribosyl-cysteine.



**FIGURE 4.15**

**HPLC SEPARATION OF STANDARD COMPONENTS**

- 1. ADP-RIBOSE**
- 2. NAD<sup>+</sup>**
- 3. AMP**
- 4. NICOTANIMIDE**



**FIGURE 4.16**

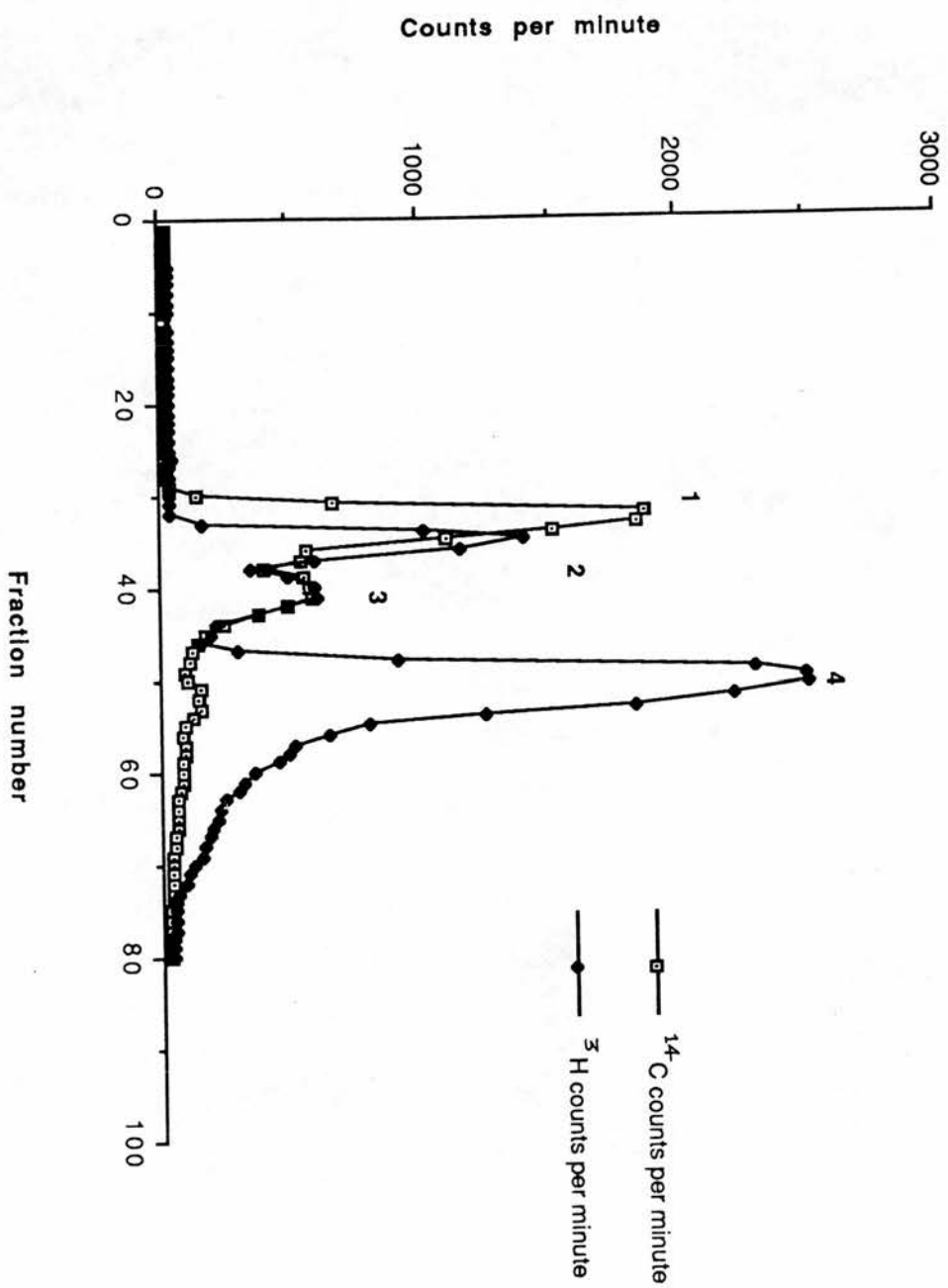
**HPLC TRACE OF  $\text{NAD}^+$ -GLYCOHYDROLASE REACTION  
PRODUCTS.**

- 1. ADP-RIBOSE**
- 2. ADP-RIBOSYL-CYSTEINE**
- 3.  $\text{NAD}^+$**

**FIGURE 4.17**

**RADIOACTIVITY INCORPORATED INTO THE  $\text{NAD}^+$ -  
GLYCOHYDROLASE REACTION PRODUCTS.**

- 1. CYSTEINE**
- 2. ADP-RIBOSE**
- 3. ADP-RIBOSYL-CYSTEINE**
- 4.  $\text{NAD}^+$**



**FIGURE 4.18**

- a. ISOLATED ADP-RIBOSYL CYSTEINE**
- b. PHOSPHODIESTERASE REACTION PRODUCTS -**
  - 1. ADP-RIBOSYL-CYSTEINE STARTING PRODUCT**
  - 2. AMP**
- c. PROPOSED REACTION MECHANISM**

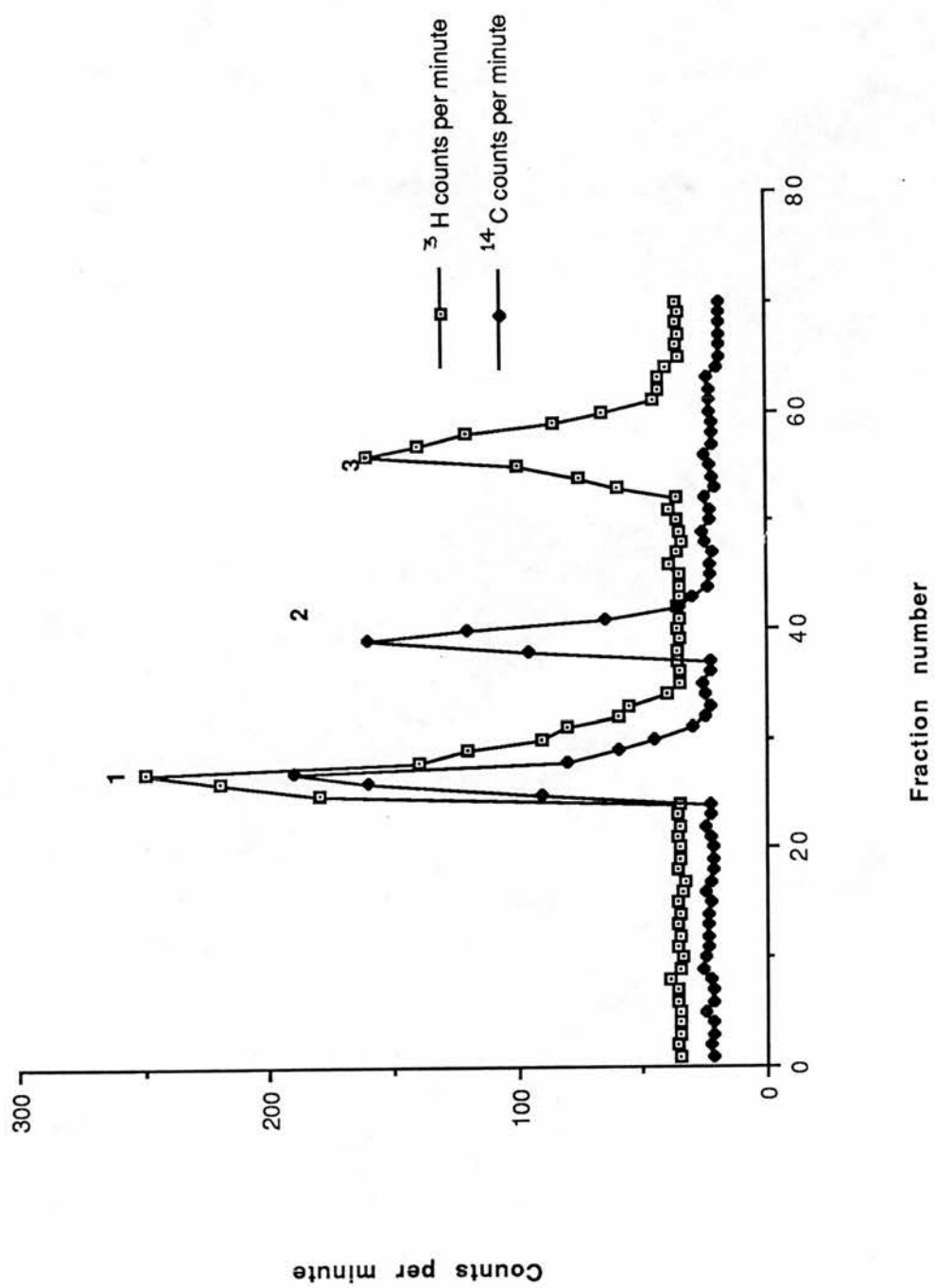




**FIGURE 4.19**

**RADIOACTIVITY INCORPORATED INTO  
PHOSPHODIESTERASE REACTION PRODUCTS.**

- 1. ADP-RIBOSYL-CYSTEINE**
- 2. PHOSPHO-RIBOSYL-CYSTEINE**
- 3. AMP**

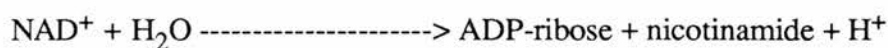


#### 4.2.7. Investigation of other putative substrates

Hurley *et al.*, (1984), suggested that the amino acid which accepts the ADP-ribose moiety from the pertussis toxin catalysed ADP-ribosylation was aspartate. Abood *et al.* (1982) and Manning *et al.*, (1984) disputed these findings and suggested that the receptor amino acid was asparagine. West *et al.*, (1985), demonstrated it was a cysteine residue within transducin which accepts ADP-ribose from the pertussis toxin catalysed ADP-ribosylation of this protein. In an attempt to determine whether the  $\text{NH}_2$  group could act as ADP-ribose acceptor from the pertussis toxin catalysed reaction, the effects of two artificial substrates designed for cholera toxin were investigated. These compounds were designed to mimic the arginine acceptor group on Gs which is covalently modified by cholera toxin catalysed ADP-ribosylation. Figure 4.20 shows the structures of arginine and asparagine and the two artificial substrates used; A603, [16-(acetylamino)decyl]guanidine and A599, [12-(phenyl methylamino)]dodecanyl guanidine. The  $\text{NAD}^+$ -glycohydrolase procedure previously described was repeated with the addition of 2 mM A603 or A599 and the results obtained are shown in Figures 4.21 and 4.22. Both artificial substrates had negligible effect on the rate of  $\text{NAD}^+$  hydrolysis indicating that neither were acting as a substrate for the toxin.

#### 4.3. DISCUSSION

The results from the experiments described in this chapter provide some additional information about the  $\text{NAD}^+$ -glycohydrolase reaction catalysed by pertussis toxin.



The rate of reaction was measured as nicotinamide release from  $\text{NAD}^+$  with adequate controls being carried out to demonstrate that the breakdown of  $\text{NAD}^+$  was an enzymic process with water acting as the acceptor of ADP-ribose. Michaelis constants

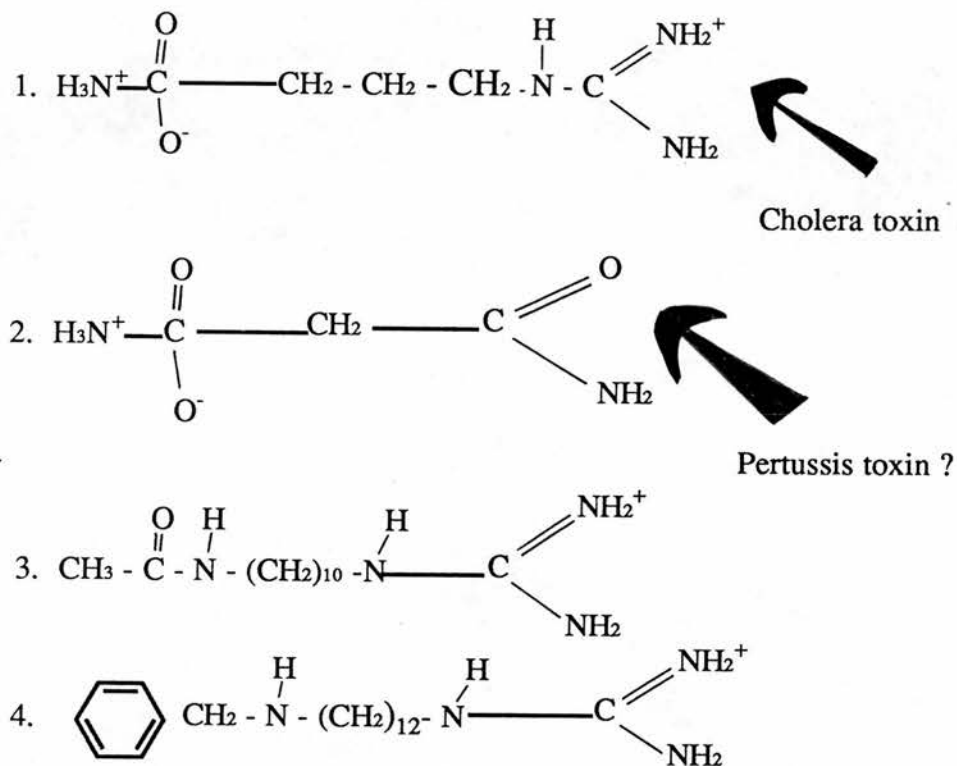
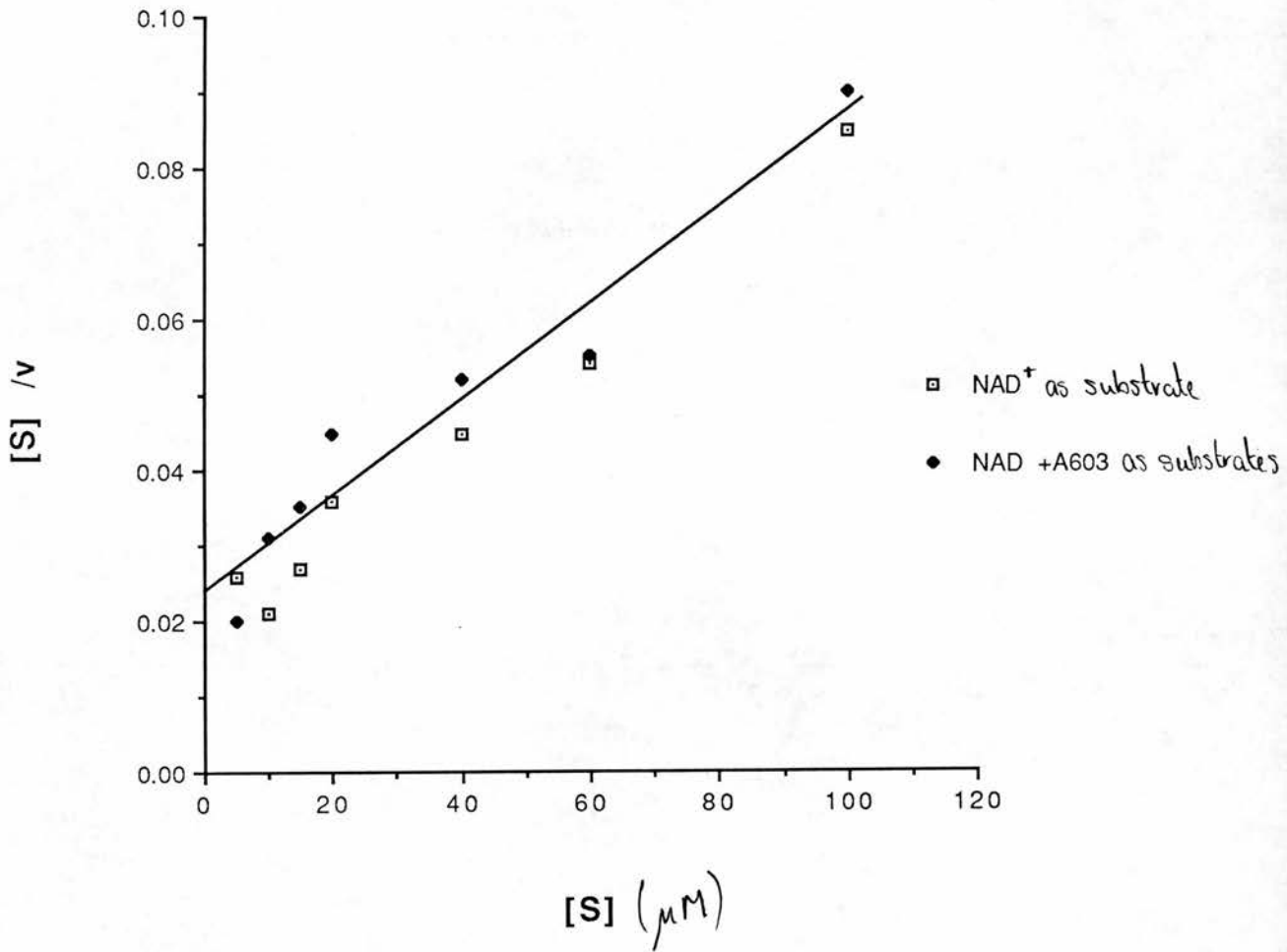


FIGURE 4.20

STRUCTURE OF ACCEPTOR COMPOUNDS FOR CHOLERA  
TOXIN CATALYSED ADP-RIBOSYLATION.

1. ARGININE
2. ASPARAGINE
3. A599
4. A603



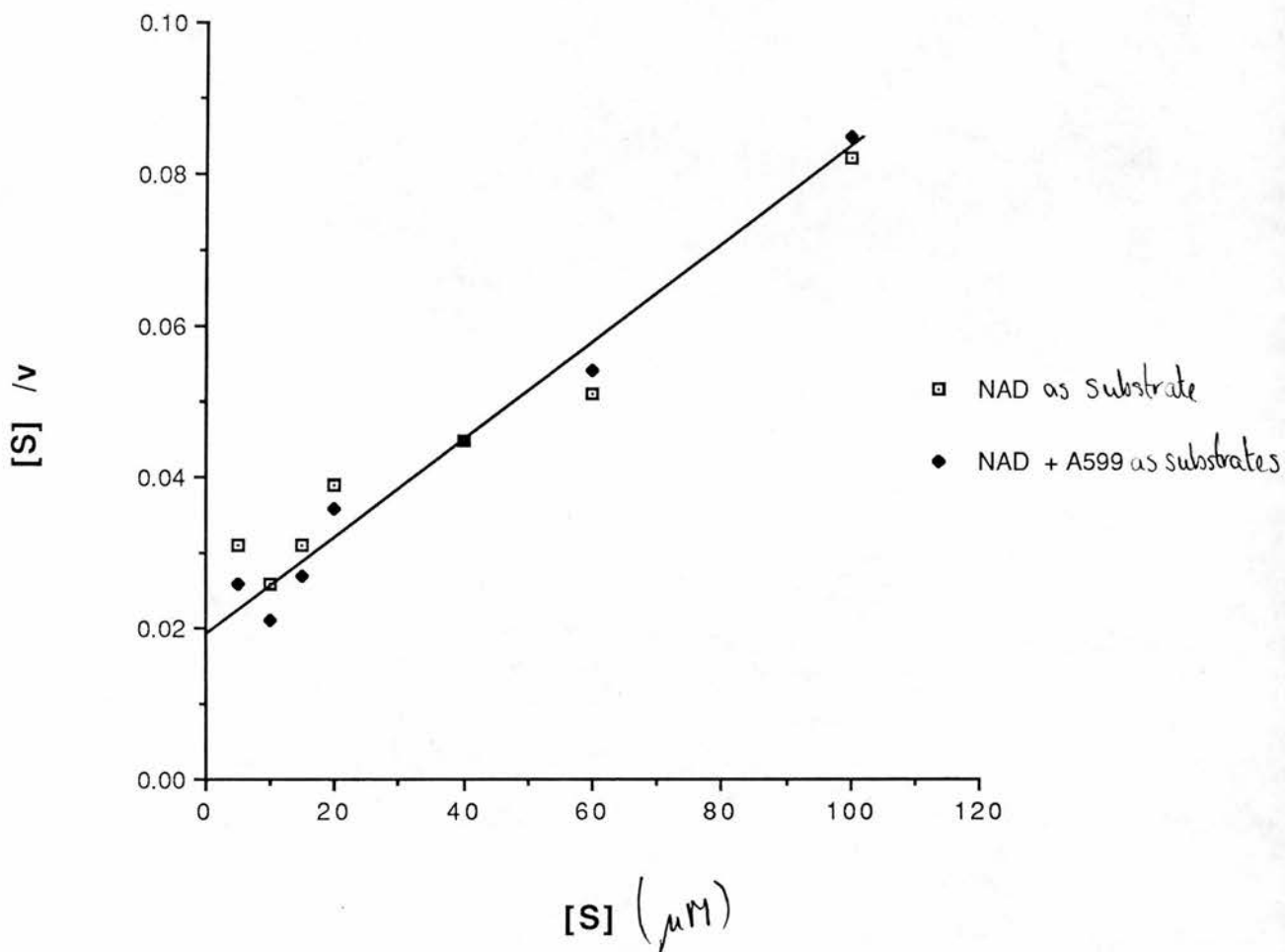
**FIGURE 4.21**

**HANES PLOT OF PERTUSSIS TOXIN CATALYSED NAD<sup>+</sup>-GLYCOHYDROLASE DEMONSTRATING THAT A603 HAS NO EFFECT ON THE REACTION PROCESS.**

$[S]/v$  = substrate concentration / pmols of nicotinamide released per hour

□ NAD<sup>+</sup> concentration

● NAD<sup>+</sup> plus 1mM A603



**FIGURE 4.22**

**HANES PLOT OF PERTUSSIS TOXIN CATALYSED NAD<sup>+</sup>-GLYCOHYDROLASE DEMONSTRATING THAT A599 HAS NO EFFECT ON THE REACTION PROCESS.**

$[S]/v$  = Substrate concentration/pmols of nicotinamide released per hour

$[S]$  = □ NAD<sup>+</sup> concentration  
 ● NAD<sup>+</sup> concentration plus 1mM A599

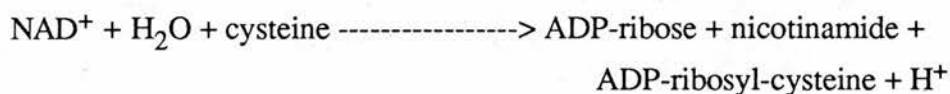
were calculated from Lineweaver-Burk, Hanes and direct linear plots. The  $K_m$  values differed slightly between plots. The Lineweaver-Burk plot is commonly used to represent kinetic data but is not recommended as it gives a misleading impression of experimental error. This plot is the double-reciprocal plot of  $1/[S]$  versus  $1/v$ . The misinterpretation of error results from small values of  $v$ , with little error in measurement leading to enormous errors in  $1/v$ , but for large values of  $v$ , these same small errors in measurement lead to barely noticeable errors in  $1/v$ . The Hanes plot, of  $[S]/v$  versus  $[S]$ , is a much better plot as over a fair range of  $[S]$  values the errors in  $[S]/v$  provide a faithful reflection of the errors in measurement of  $v$ . Finally, the direct-linear or Eisenthal and Cornish-Bowden plot of  $v$  versus  $[S]$  in which pairs of  $v$  and  $[S]$  values are joined, with each line representing one observation, provides another method of representing kinetics. If no error occurred in measurement each line representing a single determination of  $v$  at a particular value  $[S]$  would intersect at a common point, this point defining the values of  $K_m$  and  $V_{max}$ . This is very rarely the case as in nearly every experimental procedure there will be some error in measurement. The median intersection point is then used to determine  $V_{max}$  and  $K_m$ , this point being when the lines crowd closest together. The median value is used in preference to the mean as the median values avoids bias through extreme values.

The Michaelis constant of  $30 \pm 5 \mu\text{M}$  calculated from the Hanes plot for pertussis toxin catalysed  $\text{NAD}^+$ -glycohydrolase agrees well with published results (Moss and Vaughn, 1984; Burns and Mandark, 1986; Kaslow *et al.*, 1987) and was calculated at a dithiothreitol concentration of 250 mM. The  $k_{cat}$  derived from these results was  $0.25 \text{ min}^{-1}$ . This is quite a low value, as would be expected for this reaction since the  $\text{NAD}^+$ -glycohydrolase activity is secondary to ADP-ribosylation with  $\text{H}_2\text{O}$  being a poor substrate for the reaction.  $\text{NAD}^+$ -glycohydrolase only occurs in the absence of GTP-binding regulatory protein substrates.

The very high concentration of reducing agent incorporated within the assay presumably maintains the toxin in such a conformation as to maintain maximum enzymic activity throughout the duration of the assay. As previously stated the concentration of thiol required to activate cholera

toxin NAD<sup>+</sup>-glycohydrolase is 5 mM, a concentration typical of that required for enzymic activity after reduction of an essential disulphide bond. The concentration used here (250 mM) was thought to be excessive if the reducing agents were only required for enzyme activity, and their role as possible substrates was investigated. The rate of nicotinamide release was thus measured at increasing thiol concentrations (at saturating levels of NAD<sup>+</sup>) in order to determine whether these potential substrates would undergo enzyme kinetic analysis. The four sulphydryl reagents used were dithiothreitol, cysteine, mercaptoethanol and glutathione. In all cases, increasing the concentrations of these reagents increased the rate of reaction until saturation was achieved at concentrations of the order of 300 mM, suggesting that the thiols were substrates. The  $K_m$  calculated for cysteine at saturating concentrations of NAD<sup>+</sup> was 105 mM and that calculated for the other reagents were within the same range. The  $K_m$  values are very high, but the values calculated demonstrate that the thiols can be substrates at the concentrations used within the NAD<sup>+</sup>-glycohydrolase protocol (Moss *et al.*, 1983; Moss *et al.*, 1984). The order of substrate preference for pertussis toxin would be G-proteins, H<sub>2</sub>O and finally the sulphydryl group of reducing reagents.

By using NAD<sup>+</sup> and cysteine labelled with different radionucleotides the formation of a compound containing both the adenine moiety of NAD<sup>+</sup> and the carbon atoms of cysteine has been identified as a reaction product from the pertussis toxin catalysed reaction -



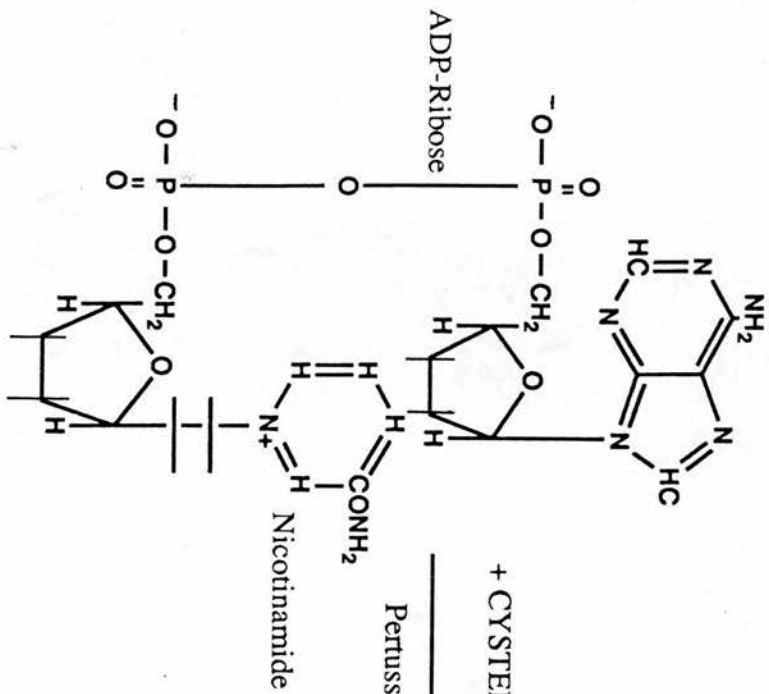
Although this does not prove a definite structure for this compound, it is reasonable to propose that the compound formed is ADP-ribosyl-cysteine with a covalent bond between the C-1 atom of ribose and the sulphur atom of cysteine as shown in Figure 4.23.

The reaction product was isolated by HPLC and treated with a snake venom



**FIGURE 4.23**

**PROPOSED REACTION MECHANISM OF ADP-RIBOSYLATION  
REACTION WITH CYSTEINE AS SUBSTRATE SHOWING THE  
PROPOSED STRUCTURE OF THE ADP-RIBOSYL-CYSTEINE  
REACTION PRODUCT.**

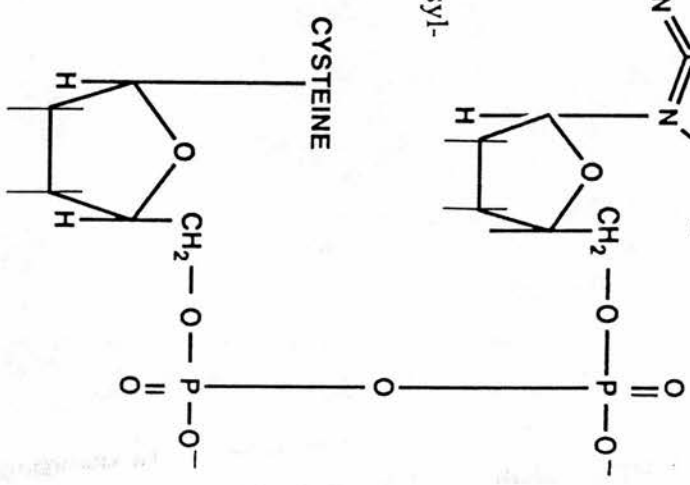


+ CYSTEINE + H<sub>2</sub>O

Pertussis toxin

NICOTINAMIDE

ADP-ribosyl-  
cysteine



phosphodiesterase which liberated AMP, as would be predicted if the reaction product was ADP-ribosyl-cysteine. Cysteine has been identified as the amino acid which accepts the ADP-ribose moiety from the pertussis toxin catalysed ADP-ribosylation of transducin by West *et al.*, 1985, who also demonstrated that the ADP-ribosyl-cysteine formed was eluted between ADP-ribose and  $\text{NAD}^+$  from a similar HPLC system, at the same position as the ADP-ribosyl-cysteine reaction product from the pertussis toxin catalysed  $\text{NAD}^+$ -glycohydrolase reaction.

These experiments show that thiols can have two roles in the activity of pertussis toxin *in vitro*: as an activator of the toxin, at lower concentrations, by reduction of a disulphide bond, and as a weak substrate for the ADP-ribosylation reaction at high concentrations.

As previously discussed it had been suggested by Abood *et al.*, (1982) and Manning *et al.*, (1984) that asparagine was the amino acid which accepts the ADP-ribose from the pertussis toxin catalysed ADP-ribosylation. It is generally believed that this residue is in fact cysteine, with the previous work discussed in this chapter adding to the evidence. However, to determine whether the  $\text{NH}_2$  group of asparagine could act as a substrate the artificial substrates designed for cholera toxin were incorporated into the reaction and their effect on the kinetics determined. The two reagents used were firstly A599, a small guanidine derivative with a polar head group, separated from a charged end group by an unknown length of carbohydrate chain (Tait and Nassau, 1984), and secondly A603 which is a similar compound having a polar head group, hydrocarbon chain and a charged end group. Both of these reagents had no effect on the pertussis toxin catalysed  $\text{NAD}^+$ -glycohydrolase reaction suggesting that the  $\text{NH}_2$  group does not act as a substrate for pertussis toxin within this *in vitro* system.

## CHAPTER 5

### BINDING OF NAD<sup>+</sup> TO PERTUSSIS TOXIN

#### 5.1. INTRODUCTION

Binding of ligand to protein is the primary step in all enzyme catalysed reactions. Once the substrate ligand has bound to the enzyme, the enzyme can launch its catalytic conversion. In chapter 4 a  $K_m$  of  $30 \pm 5 \mu M$  was calculated for the NAD<sup>+</sup>-glycohydrolase activity of pertussis toxin. This implies that the binding affinity of the toxin for NAD<sup>+</sup> should be of the same order of magnitude as the  $K_m$ . Many experimental methods have been devised for measuring ligand-protein binding. For soluble protein - ligand systems the method most extensively used is equilibrium dialysis (Osbourne, 1906).

The number of binding sites on S-1 for NAD<sup>+</sup> has not been measured. It is reasonable to suppose that the toxin has one binding site for NAD<sup>+</sup> as it seems unlikely that the protein would have evolved separate catalytic sites for the NAD<sup>+</sup>-glycohydrolase and ADP-ribosyltransferase activities, as they are essentially the same reaction. There is also no evidence for any replication in amino acid sequence indicative of two or more NAD<sup>+</sup>-binding sites, however, this one binding site assumptions needs to be confirmed. Poly (ADP-ribosyl) transferase enzymes isolated from the eukaryote nucleus have been shown to contain both an initial catalytic site and one or more different acceptor sites (Bauer and Kun, 1984; Kawaichi *et al*, 1981). These reactions involve the binding of two or more molecules of NAD<sup>+</sup> resulting in the formation of a poly-ADP-ribose molecule.

The stoichiometry of binding must be determined in order to characterise any binding interaction. The maximum number of moles of ligand bound per mole of protein ( $n$ ), and the equilibrium dissociation constant  $K_d$  were calculated in this chapter

by the techniques of equilibrium dialysis and quenching of intrinsic fluorescence. The mathematical description of equilibrium in the simplest case of protein P and ligand L is given by :



$$K_d = \frac{[P][L]}{[PL]}$$

1.

If the number of moles of L bound per mole of protein P is defined as n then :-

$$n = \frac{\text{concentration of [L] bound to protein}}{\text{total concentration of all forms of P}}$$

$$= \frac{[P][L]}{[P] + [PL]}$$

now from equation 1.  $[PL] = \frac{[P][L]}{K_d}$

$$n = \frac{\frac{[P][L]}{K_d}}{[P] + \frac{[P][L]}{K_d}}$$

$$n = \frac{[L]}{K_d + [L]} \quad 2.$$

The above equations apply only in the case when  $n \leq 1$  and is the fractional saturation of sites. This model assumes that one of P can bind one mole of L and that there is no cooperation on binding of substrate.

Two types of experimental procedures have been used to calculate the stoichiometry of  $\text{NAD}^+$ -binding to pertussis toxin. Firstly a separation method in which the protein-ligand system is physically separated from free ligand at equilibrium. This equipment enables direct measurements of bound ligand at equilibrium to be made without disturbing the system. Equilibrium dialysis is the method used in this case. Equilibrium can be measured accurately using this method as free ligand L and

fractional saturation,  $n$  can be measured independently.

The second method involves the application of some measurable change in protein and ligand characteristics on binding of ligand to protein. The most commonly used techniques are spectroscopic measurements of changes in absorbance, fluorescence or optical rotary dispersion. These techniques are very sensitive and can provide information on both  $K_d$  and the nature of the protein-ligand complex rapidly and without disturbing the equilibrium of the system.

## 5.2. RESULTS

### 5.2.1. The principles of equilibrium dialysis

Within the equilibrium dialysis chamber a solution of protein on one side of a semi-permeable membrane is allowed to reach equilibrium with ligand in the same solvent on the other side (Figure 5.1). At equilibrium the chemical potential of free ligand  $[L]$  must be the same on both sides of the membrane. Protein concentration should be sufficiently high to enable direct measurement of bound ligand, but not excessively high as this would result in free ligand depletion and could disrupt the equilibrium of the system by setting up a potential gradient causing a loss of free ligand in the protein chamber. If the amount of ligand in the system is greater than the amount of protein this should not be a problem. The total amount of ligand added to the system is known and the free ligand concentration can be calculated from the system, then the amount of ligand bound to protein ( $n$ ) can be calculated. This can be shown mathematically as :

$$n = \frac{L_p}{P_T} \quad 3.$$

When  $L_p$  is the number of moles of ligand bound to protein.

$P_T$  is the total number of moles of protein.

$L_T$  is the total ligand.

$[L]$  is free ligand then:

$$L_p = L_T - V_T [L] \quad 4.$$

Where  $V_T$  is the total volume of solution in both compartments.

In most situations some free ligand will bind to the dialysis apparatus -  $L_M$  therefore  $L_p$  is expressed as:

$$L_p = L_T - V_T [L] - L_M \quad 5.$$

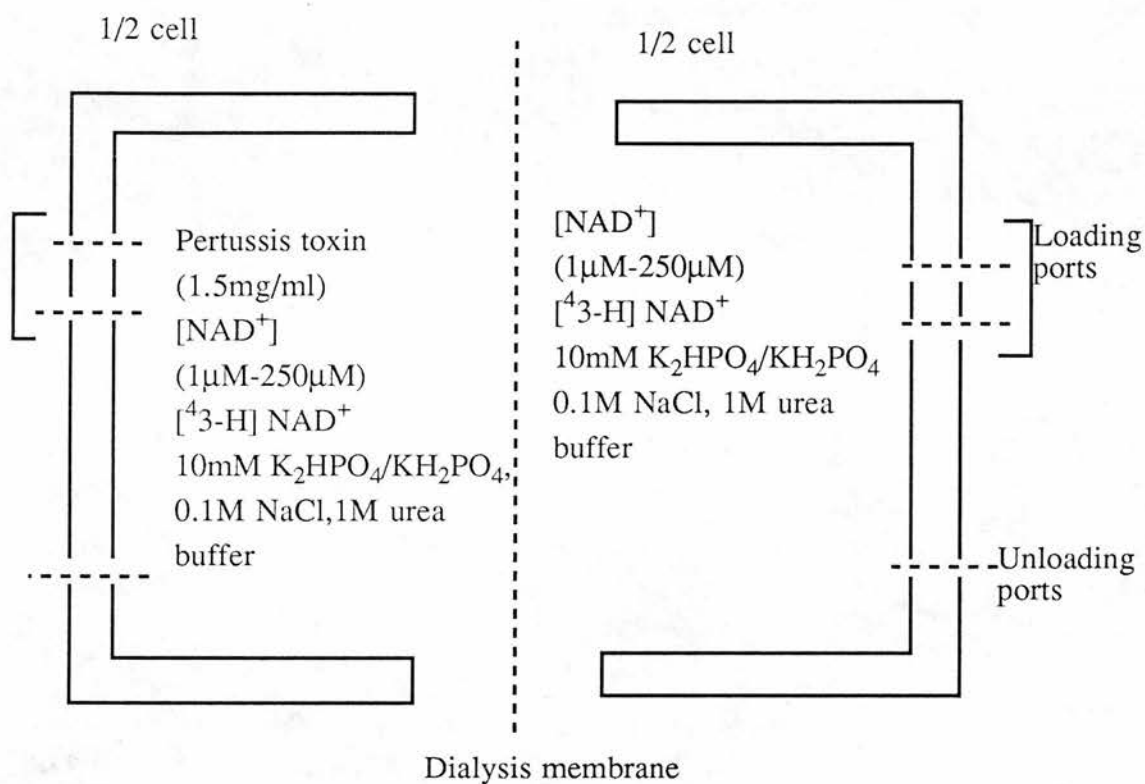
If necessary correction must be made for  $L_M$  in calculations.

As protein itself carries a net charge, and the ligand of interest in this system,  $NAD^+$ , also carries a net charge, then even in the absence of binding the concentration of free ligand may not be the same in both compartments because of the Donnan effect (Donnan, 1924). This difference in concentration can however be made negligible by ensuring that the buffer used within the system is of a sufficiently high ionic strength. At high protein concentrations, relatively high concentrations of a supporting electrolyte may be required within the system to render the Donnan effect negligible.

Equilibrium dialysis was carried out using a Dianorm equilibrium dialysis apparatus. This system consists of an array of teflon cells in a rotating assembly. Each cell is made up of two half cells, which are separated by a dialysis membrane, with ports on both sides for the loading and unloading of samples. This apparatus allows a quick and reproducible estimates of both  $K_d$  and  $n$  (Weber *et al.*, 1960). A number of preliminary experiments were performed to characterise the system in terms of equilibrium time, stability of protein and ligand etc., and these are described below.

### 5.2.2. Dialysis time

The time taken to reach equilibrium under the conditions outlined in section 2.2.3 was determined by the time taken for solutions of  $[4\text{-}^3\text{H}]\text{NAD}^+$  (20 nCi) containing 5-200  $\mu\text{M}$  unlabelled  $\text{NAD}^+$  to equilibrate between the two half-cells at



**FIGURE 5.1**  
**EQUILIBRIUM DIALYSIS SYSTEM**



regular time intervals over a period of 5 hours at 30°C, and the radioactivity counted. Equilibrium was reached after 180 minutes as shown in Figure 5.2.

### 5.2.3 Recovery of radioactivity

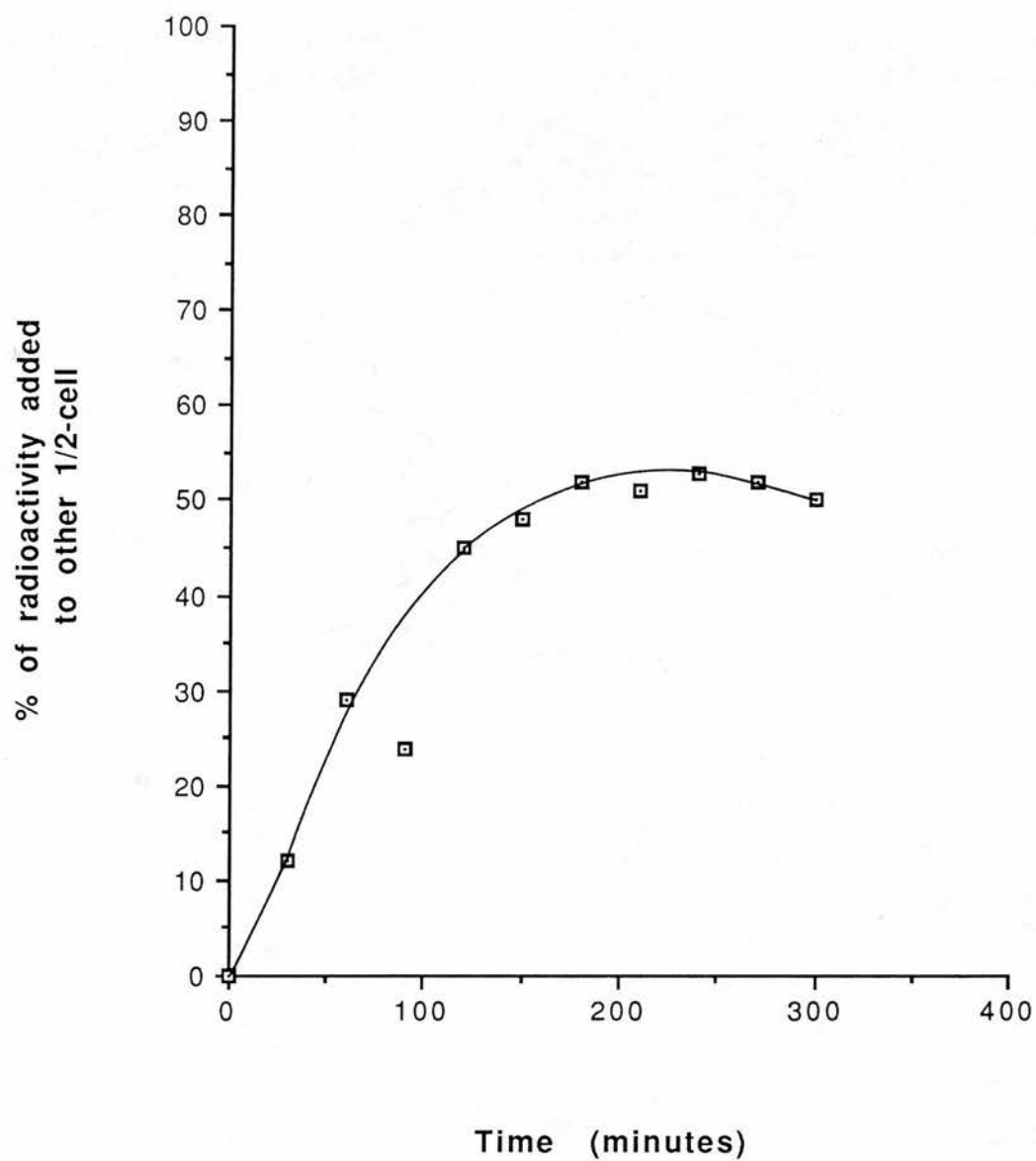
When  $[4\text{-}^3\text{H}]\text{NAD}^+$  was dialysed against pertussis toxin (1.5 mg/ml) comparison of the total amount of radioactivity added with the amounts found in 100  $\mu\text{l}$  aliquots taken from each half cell during dialysis, showed that more than 95% of the total radioactivity was recovered. The small loss of radioactivity was most probably caused by ligand binding to the dialysis membrane and the teflon cell.

### 5.2.4. Non-specific breakdown of $\text{NAD}^+$

To determine the integrity of  $\text{NAD}^+$  after dialysis against pertussis toxin, samples were removed at the end of each experiment and were analysed by paper chromatography as described in section 2.2.3.3. In all cases less than 5% of the  $\text{NAD}^+$  had broken down to nicotinamide. This indicates that unlike diphtheria toxin, which can completely hydrolyse a ten fold molar excess of  $\text{NAD}^+$  within two hours (Kandel *et al*, 1974), the  $\text{NAD}^+$ -glycohydrolase activity of pertussis toxin should not complicate the dialysis experiments.

### 5.2.5 Binding experiments

Experiments were carried out using pertussis toxin at concentrations in excess of 1 mg/ml ( $1 \times 10^{-9}$  moles <sup>in</sup> 200  $\mu\text{l}$ ) and 1 - 200  $\mu\text{M}$   $\text{NAD}^+$  containing 20 nCi  $[4\text{-}^3\text{H}]\text{NAD}^+$ . A high protein concentration was used so that sufficient ligand was bound to the protein, enabling accurate measurements of free ligand concentration and therefore reducing the error in calculation. High protein concentration was obtained by using a



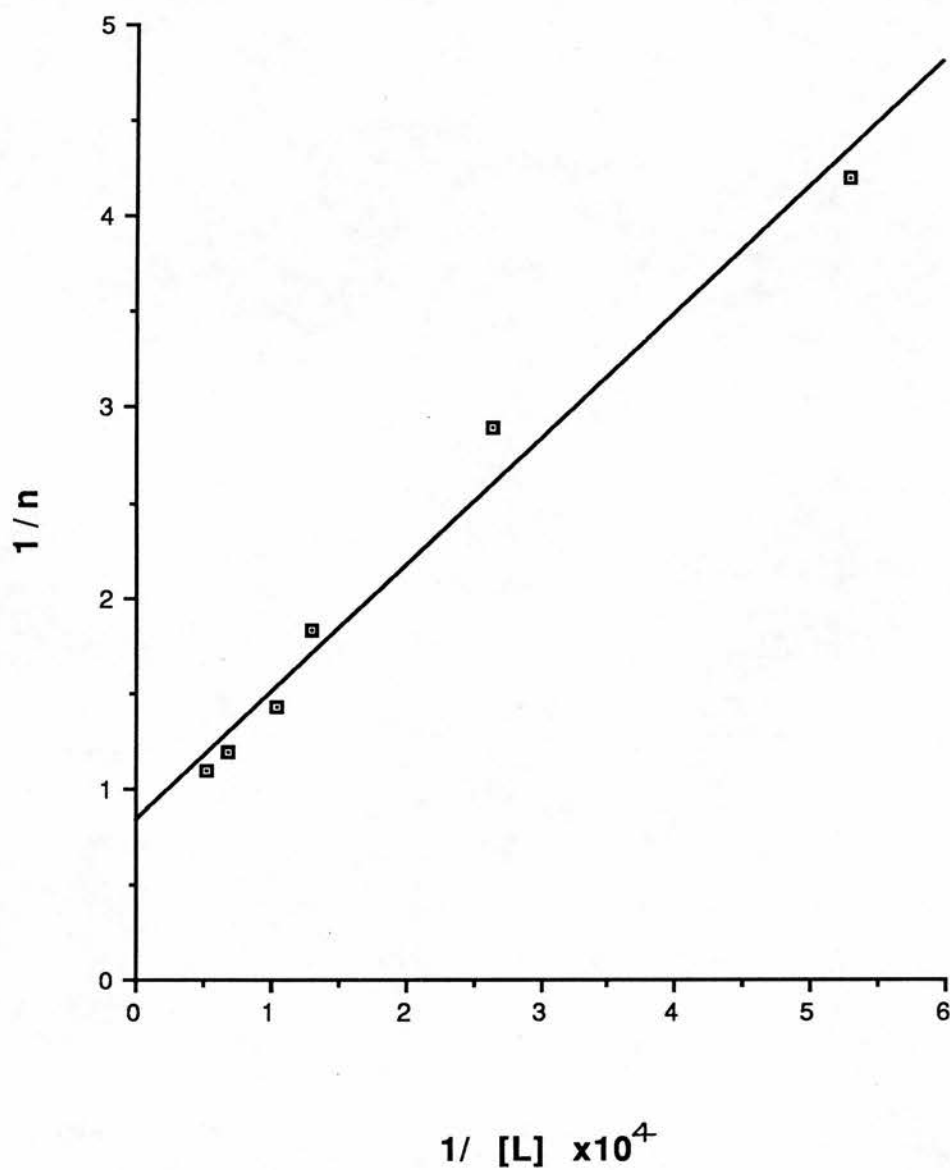
**FIGURE 5.2**

**PRELIMINARY EXPERIMENT FOR DETERMINATION OF TIME  
TAKEN FOR EQUILIBRIUM DIALYSIS SYSTEM TO REACH  
EQUILIBRIUM.**

buffer containing 1 M urea. The urea was necessary as pertussis toxin is notoriously insoluble in most aqueous systems. As will be discussed in a later section in this chapter, circular dichroism analysis of the toxin demonstrates that the structure of the toxin is unaffected in this solution, therefore urea at this concentration should not affect binding. The buffer used was 0.1M NaCl, 0.01M  $K_2HPO_4$ , pH 7.2, 1M urea. The relatively high concentration of supporting electrolyte, NaCl, ensures that the Donnan effect is negligible within this system (van Holde, 1971).

Experiments were carried out at a number of different temperatures; 7°C, 23°C, 30°C, 37°C, 40°C and 50°C. Typical graphs are shown in Figures 5.3- 5.7 and also in Appendix I as plots of  $1/n$  versus  $1/[L]$ , double reciprocal plots of moles of ligand bound per mole of protein ( $n$ ) versus free ligand concentration ( $n$ ) (Hughes-Klotz plot), being used in preference to the more familiar Scatchard plot (Scatchard, 1949). Logarithmic plots demonstrating saturation are shown in Figures 5.8 and 5.9.

The binding parameters are summarised in Table 5.1 and from these results at different temperatures some basic thermodynamic parameters could be obtained. The van't Hoff Isochore shown in Figure 5.10 gives calculated enthalpy change  $\Delta H$  of 30 kJoules.  $\text{mol}^{-1}$  for the binding of  $NAD^+$  to pertussis toxin. This is a typical value for an enzyme-ligand interaction.



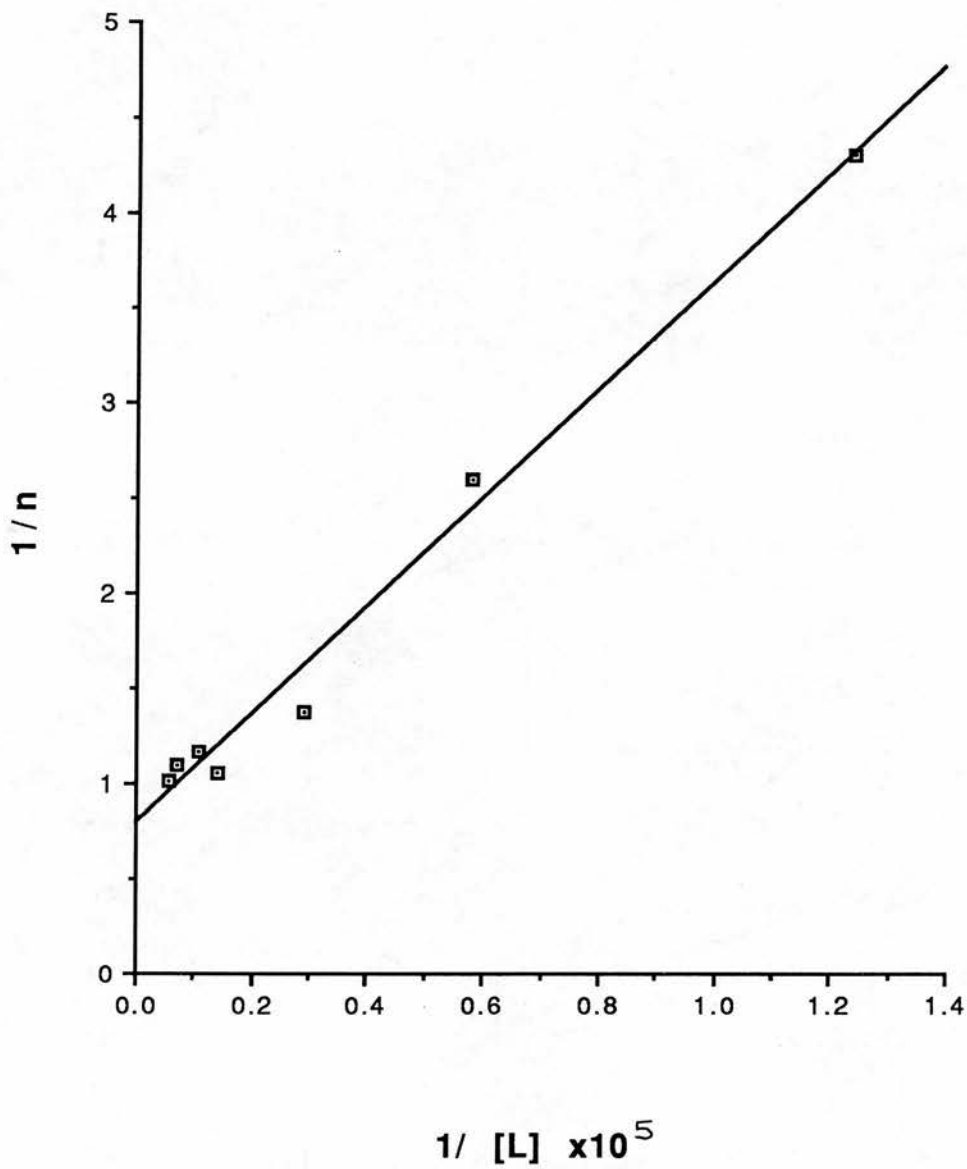
**FIGURE 5.3**

**EQUILIBRIUM DIALYSIS SYSTEM AT 7°C**

$$K_d = 90 \pm 5 \mu\text{M}$$

$1/n$  =  $1/\text{moles of ligand bound per mole of protein}$

$1/[L]$  :  $1/\text{free ligand } [NaO^+]\text{ concentration}$



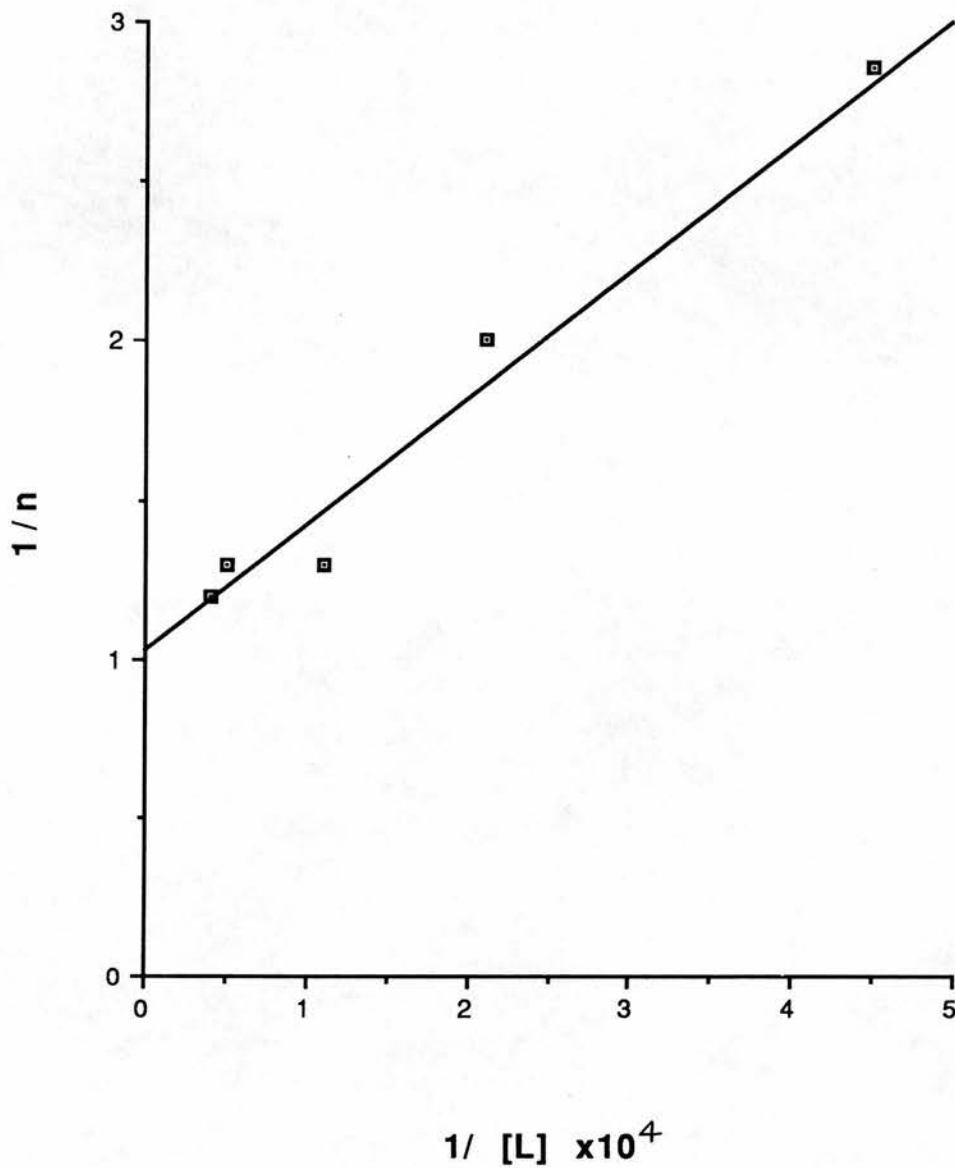
**FIGURE 5.4**

**EQUILIBRIUM DIALYSIS AT 20°C**

$$K_d = 40 \pm 1 \mu M$$

$1/n$  = 1/number of moles of ligand bound per mole of protein

$1/[L]$  = 1/free ligand [NAB'] concentration



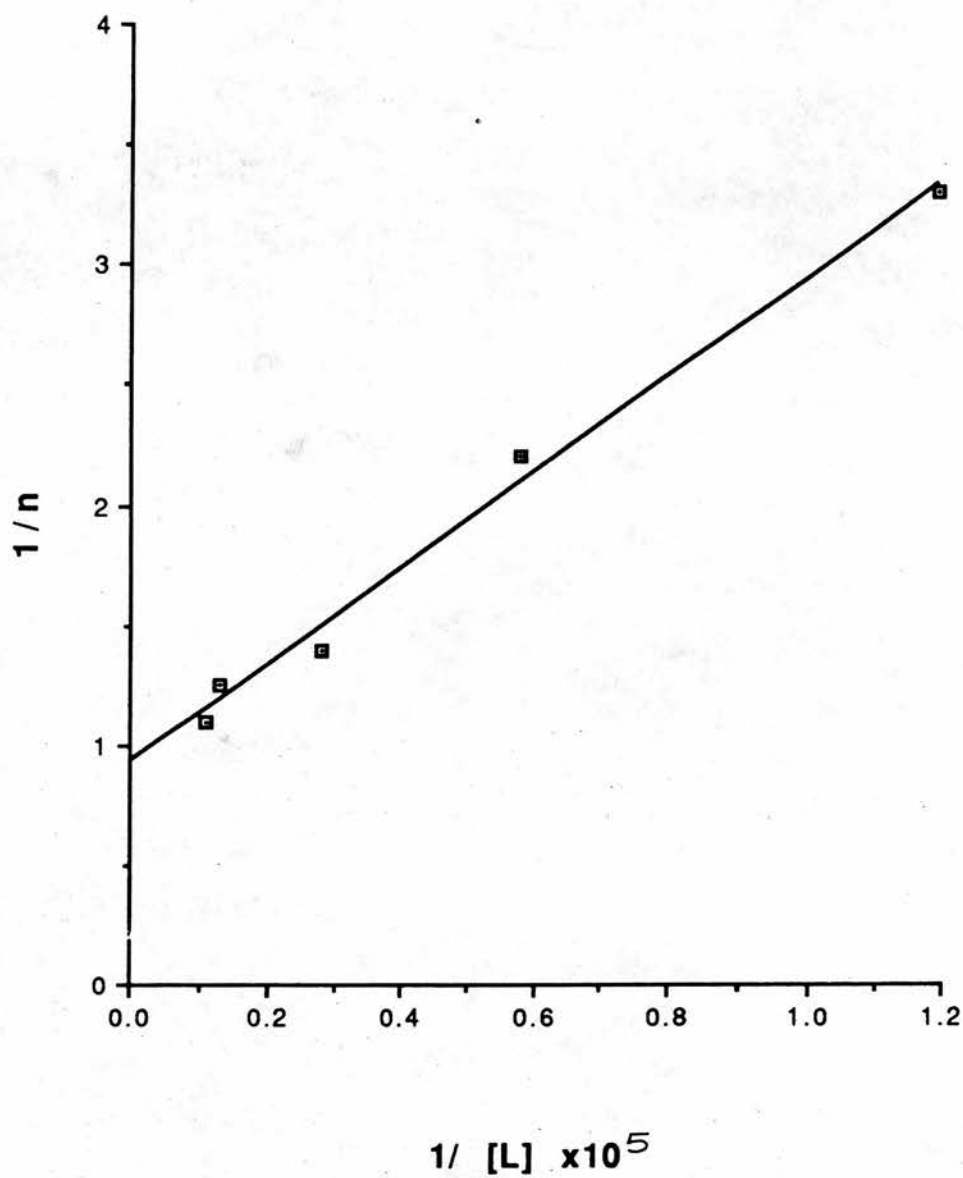
**FIGURE 5.5**

**EQUILIBRIUM DIALYSIS AT 23°C**

$$K_d = 34 \pm 2 \mu\text{M}$$

$1/n$  =  $1/\text{number of moles of ligand bound per mole of protein}$

$1/[L]$  =  $1/\text{free ligand [NAD}^+] \text{ concentration}$



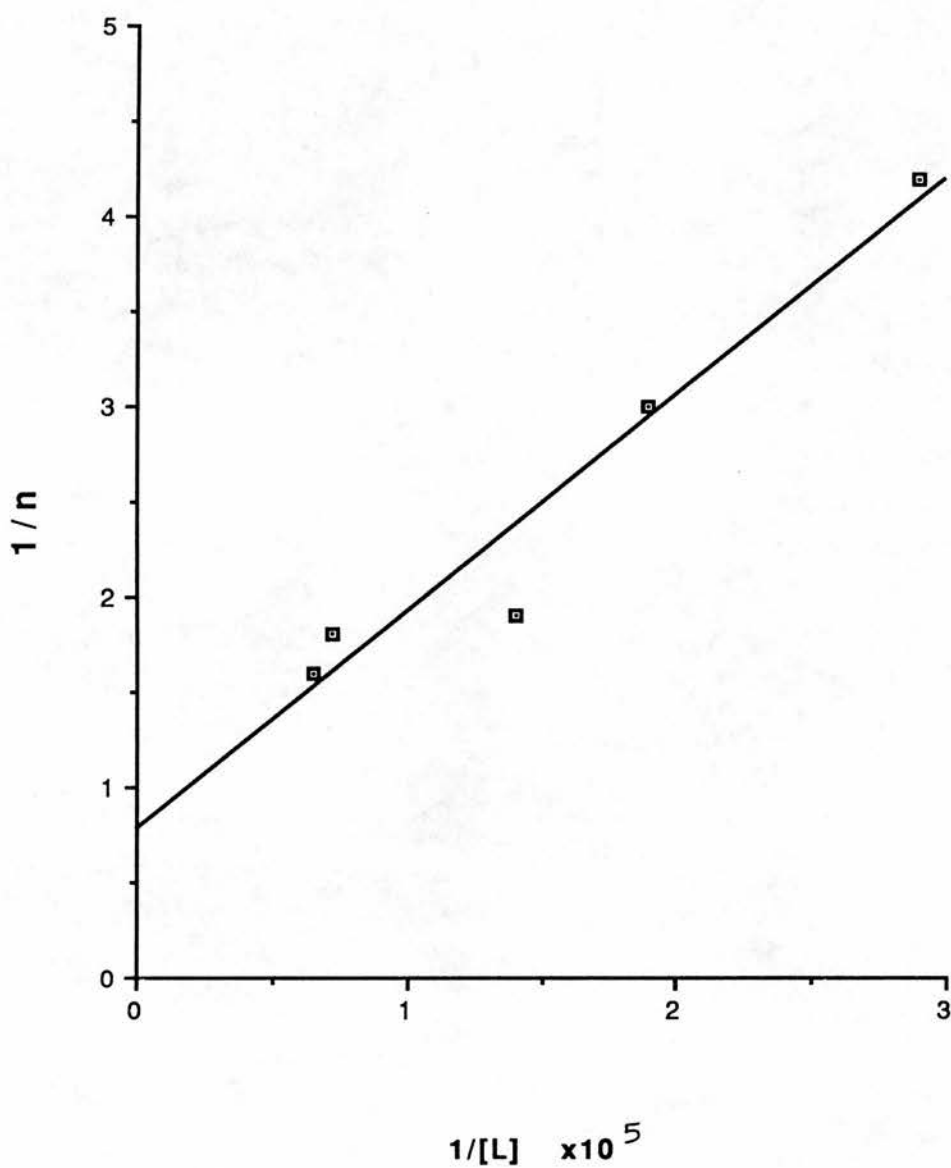
**FIGURE 5.6**

**EQUILIBRIUM DIALYSIS AT 30 °C**

$$K_d = 24 \pm 3 \mu\text{M}$$

$1/n$  =  $\frac{1}{\text{number of moles of ligand bound per mole of protein}}$

$1/[L]$  =  $\frac{1}{\text{free ligand [NAD}^+] \text{ concentration}}$



**FIGURE 5.7**

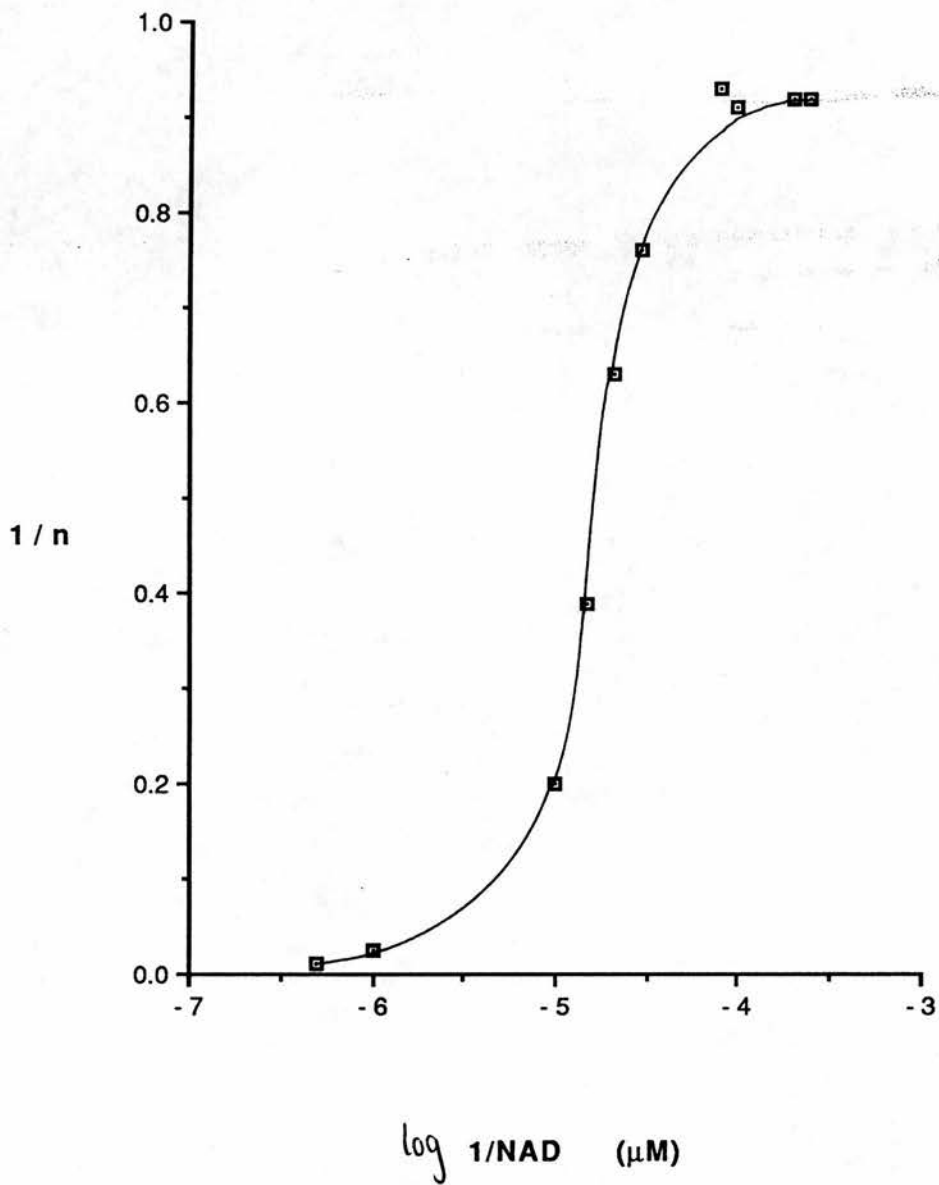
**EQUILIBRIUM DIALYSIS AT 40°C**

**K<sub>d</sub> = 18 μM**

$1/n$  = 1/number of moles of ligand bound per mole of protein

$1/[L]$  = 1/free ligand [NAD<sup>+</sup>] concentration



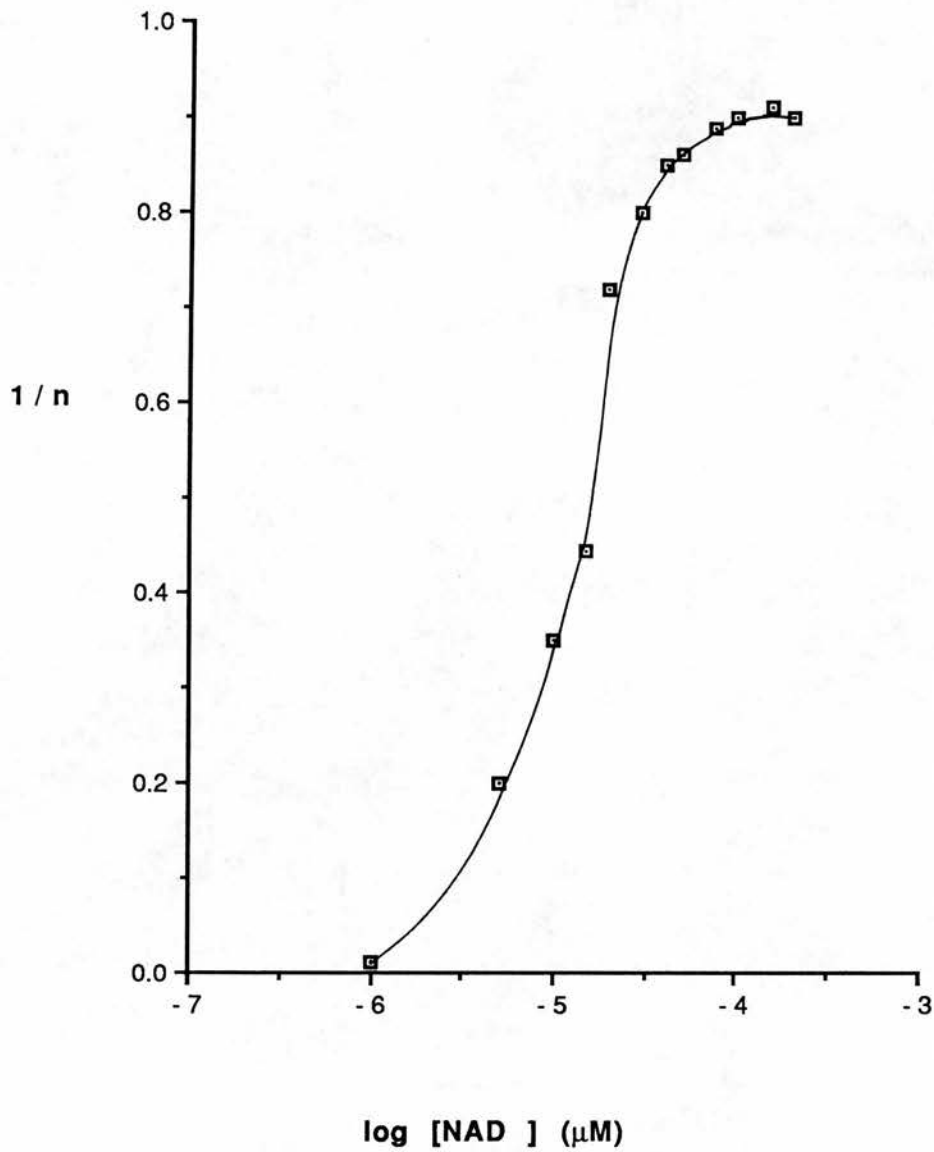


**FIGURE 5.8**

**LOGARITHMIC PLOT OF  $NAD^+$ -BINDING TO PERTUSSIS  
TOXIN AT  $20^\circ C$**

**$K_d$  AT THE POINT OF INFLECTION =  $40 \mu M$**

$1/n$  =  $\frac{1}{2}$  number of moles of ligand bound per mole of protein

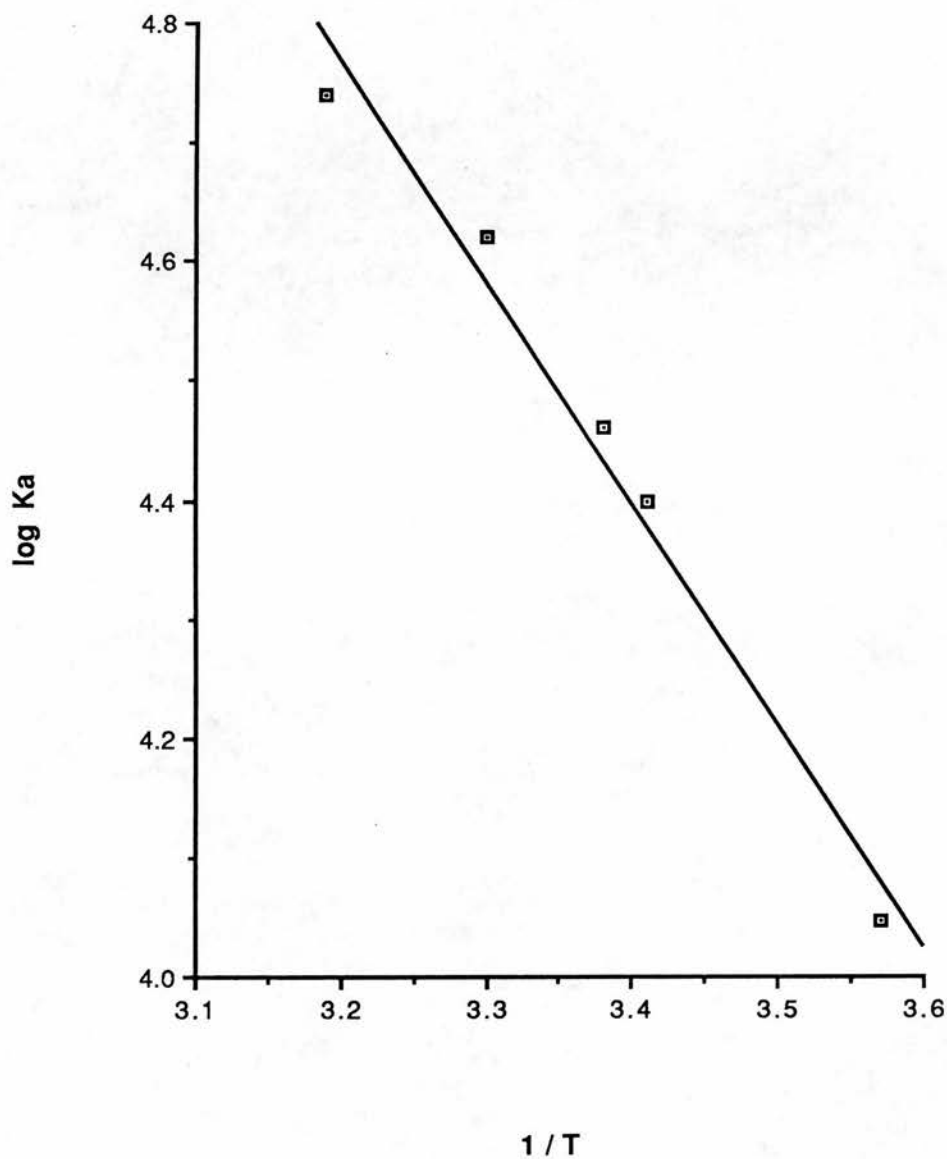


**FIGURE 5.9**

**LOGARITHMIC PLOT OF NAD<sup>+</sup>-BINDING TO PERTUSSIS  
TOXIN AT 30°C**

**K<sub>d</sub> AT THE POINT OF INFLECTION = 25 μM**

*1/n = 1/number of moles of ligand bound per mole of protein*



**FIGURE 5.10**

**van't HOFF ISOCHORE FOR NAD<sup>+</sup>-BINDING TO PERTUSSIS  
TOXIN AT 30°C**

$$\Delta H = 30 \text{ kJoules.mol}^{-1}$$

$$\log K_a = \log 1/K_d \text{ (dissociation constant)}$$

$$1/T = 1/\text{temperature (Kelvin)}$$

**TABLE 5.1**            **Binding parameters calculated by equilibrium dialysis and fluorescence.**

**1.    Equilibrium dialysis**

Temperature	Kd ( $\mu$ M)	No. of binding sites (n)
7°C	$90 \pm 5$ S.D.	$1.15 \pm 0.05$ S.D.
20°C	$40 \pm 5$ S.D.	$1.10 \pm 0.05$ S.D.
23°C	$35 \pm 2$ S.D.	$1.10 \pm 0.20$ S.D.
30°C	$24 \pm 2$ S.D.	$1.00 \pm 0.10$ S.D.
40°C	$18 \pm 1$ S.D.	$1.00 \pm 0.15$ S.D.

**2.    Fluorescence**

20°C	43 $\mu$ M
30°C	28 $\mu$ M
40°C	20 $\mu$ M

---

**5.2.6.        Quenching of protein fluorescence by NAD<sup>+</sup>**

Fluorescence spectroscopy was an alternative approach used to study this toxin-ligand interaction. This method was previously used by Kandel *et al.*, (1974) and by Chung and Collier, (1977) to study the binding of NAD<sup>+</sup> to diphtheria toxin fragment A and *Pseudomonas aeruginosa* exotoxin A respectively. The binding of NAD<sup>+</sup> causes a quenching of intrinsic fluorescence (excitation 285, emission 333 nm) of both these toxins. The intrinsic fluorescence of diphtheria toxin fragment A was reduced by up to

60% while that of exotoxin A was reduced by 20%. In both cases the concentration of  $\text{NAD}^+$  which gave half maximal quenching was about  $9 \mu\text{M}$ , a value which correlated well with the  $K_m$  values of  $8 \mu\text{M}$  calculated for the  $\text{NAD}^+$ -glycohydrolase activities of both toxins. This quenching is thought to result from formation of a charge transfer complex between the nicotinamide moiety of  $\text{NAD}^+$  and a tryptophan residue within each of the toxins.

A catalytically active peptide of pertussis toxin S-1 subunit, the C180 peptide, has been engineered to facilitate investigation into the catalytic site of the protein (Barbieri *et al.*, 1989; Cortina and Barbieri, 1989). Site directed mutagenesis of Trp26 was carried out with the tryptophan residue being replaced with aspartic acid or asparagine. The substituted peptides were assayed for  $\text{NAD}^+$ -glycohydrolase activity and the rate of  $\text{NAD}^+$  hydrolysis was less than 1% of the native C180 peptide. Phenylalanine and tyrosine substitution gave smaller reductions in the rates of  $\text{NAD}^+$  hydrolysis, 6 - 20% of that of the native peptide respectively. This tryptophan residue appears to have an analogous function to the tryptophan residues involved in the efficient binding of  $\text{NAD}^+$  to diphtheria toxin fragment A and *pseudomonas aeruginosa* exotoxin A, which suggests that the intrinsic fluorescence of pertussis toxin could be used to determine its  $\text{NAD}^+$  binding parameters.

When pertussis toxin is excited at 285 nm, the emission spectrum shows two peaks, one at 307 and the other at 338 nm. The S-1 subunit has been shown to contain 18 tyrosine residues (Locht and Keith, 1986; Nicosia *et al.*, 1986). Tyrosine residues fluoresce at  $\approx 305$  nm when excited at 280 nm, suggesting that the first peak is as a result of tyrosine fluorescence. The maximum at 338 nm therefore corresponds to tryptophan fluorescence. Titrations were carried out at 280 nm excitation and 333 nm emission (see Figure 5.11 for fluorescence difference spectra of 10 mM potassium phosphate, 0.1 M NaCl pH 7.2 buffer versus 0.1 mg/ml pertussis toxin in the same buffer between 290 and 380 nm. )

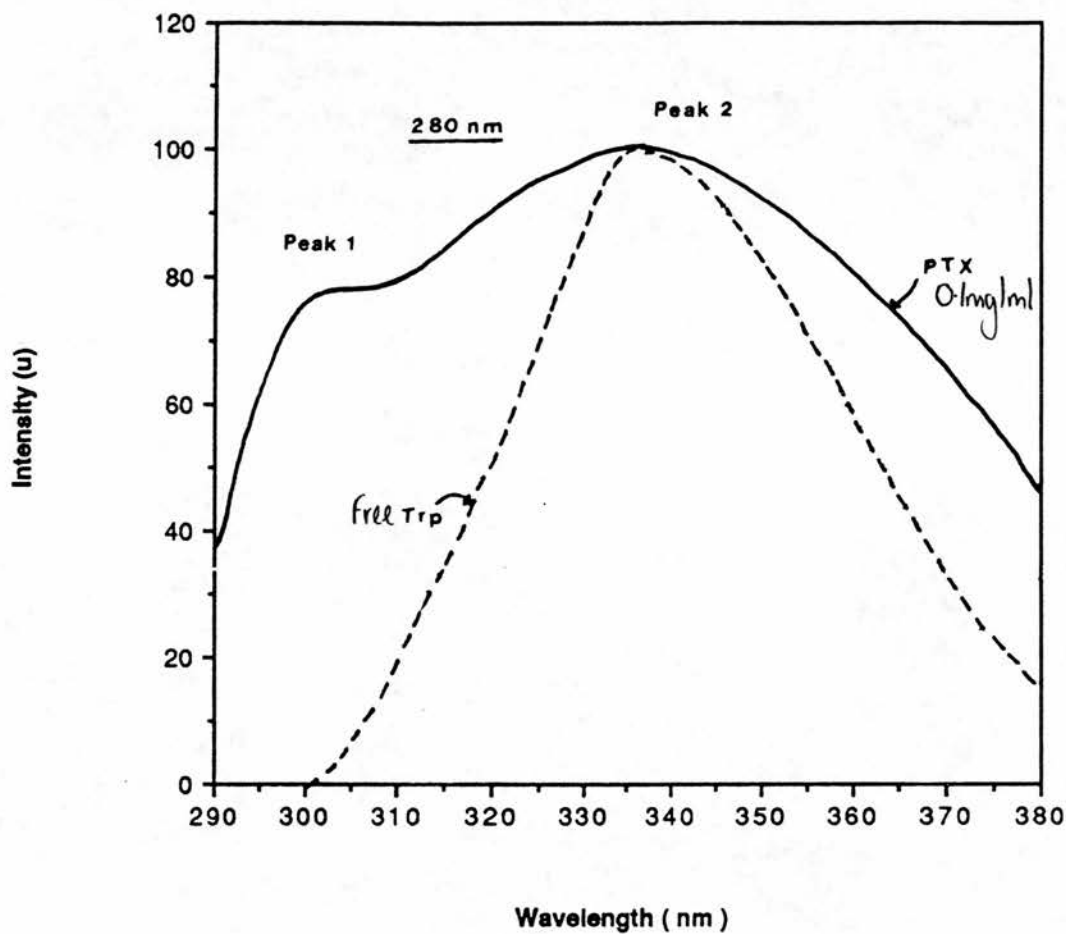


FIGURE 5.11

FLUORESCENCE DIFFERENCE SPECTRA OF 10mM POTASSIUM PHOSPHATE, 0.1M NaCl pH7.2 versus 0.1m PERTUSSIS TOXIN IN THE SAME BUFFER BETWEEN 290 AND 380nm.

THE EMISSION SPECTRA OF 10 $\mu$ M FREE TRYPTOPHAN IS ALSO SHOWN.....

### 5.2.7. Fluorescence results

In equilibrium dialysis free ligand concentration and protein-ligand complex could be measured directly at equilibrium. From these values the fractional saturation,  $n$ , was determined. Fluorescence, on the other hand, provides an indirect method of determining fractional saturation. In this case we have assumed that the reduction in fluorescence at 280 nm excitation, 333 nm emission can be correlated to  $n$ , a valid assumption as titrations show saturation at higher concentrations of  $\text{NAD}^+$  and the values derived for  $K_d$  are in close agreement with those from equilibrium dialysis. This technique for determining binding parameters assumes that it is only the protein which fluoresces at the wavelengths involved. This is not the case and correction factors have been calculated to take into account the contribution made by  $\text{NAD}^+$  (inner filter effects). This has been done by two methods. Firstly, a solution of tryptophan, of the same initial fluorescence as the toxin solution, was titrated with  $\text{NAD}^+$  and secondly, by the method of Honore and Pedersen (1989). This second method involved the following correction factor:

$A_{\text{EMMIS}}$  = Absorbance 285 nm

$A_{\text{ECIT}}$  = Absorbance 333 nm

$$F_{\text{CORR}} = F_{\text{OBS}} \times \text{Antilog} \left( \frac{A_{\text{EMMIS}} + A_{\text{ECIT}}}{2} \right)$$

and accounts for the contribution made by UV absorption at the emission and excitation wavelengths. Fractional saturation was then calculated as follows:

$$n = \frac{[L]}{K_d + [L]}$$

from section 5.1.1.

2.

rearrangement gives:

$$n = 1 + \frac{K_d}{[L]}$$

A plot of  $1/n$  versus  $1/[L]$  should give a straight line with slope  $K_d$ . The change in relative fluorescence intensity can be substituted for  $n$  as shown in the following equation:

$$\frac{1}{f} = \frac{1}{f_{\max}} + \frac{K_d}{[L]} \quad 6.$$

where  $f_{\max}$  is the maximum change in relative fluorescence intensity when toxin is saturated with  $\text{NAD}^+$  is the change in relative fluorescence intensity at a particular concentration of  $[L]$ .

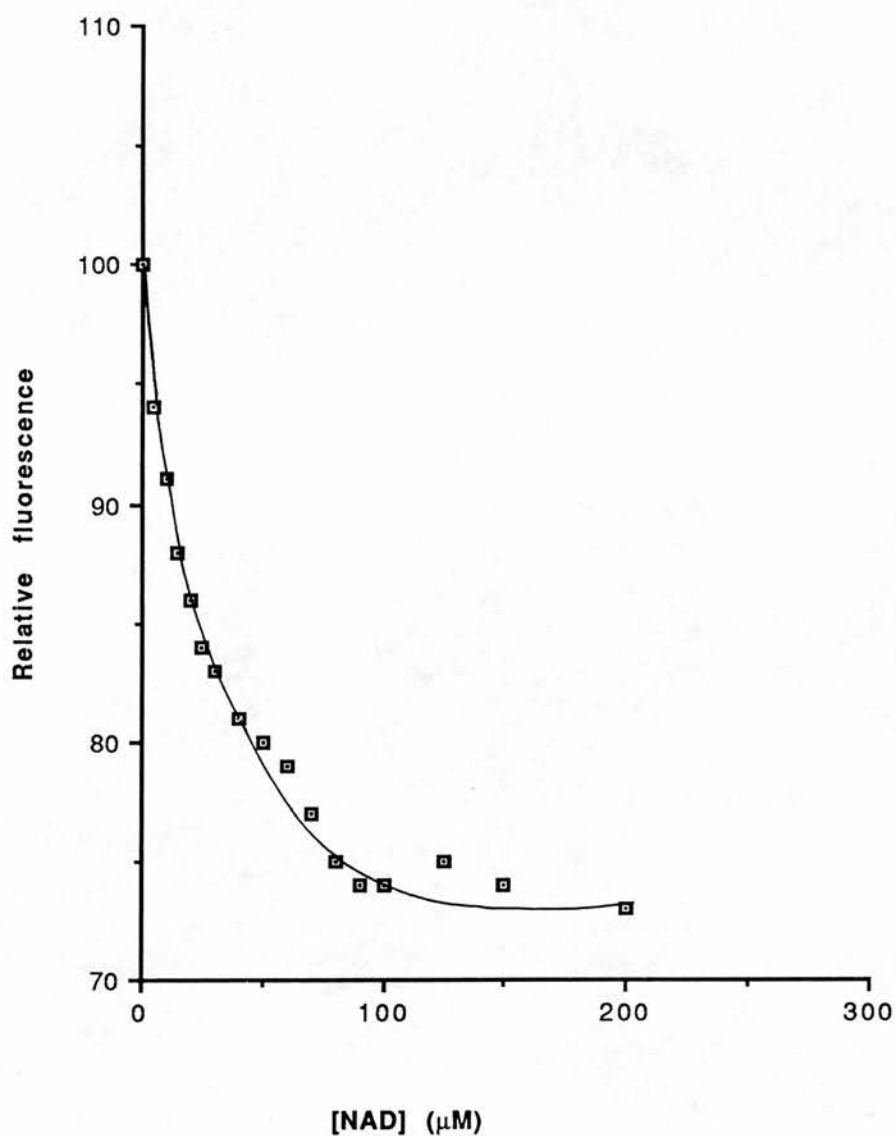
Titration were carried out as outlined in section 2.2.3.10. at  $20^\circ\text{C}$ ,  $30^\circ\text{C}$  and  $40^\circ\text{C}$ , the results are shown in Figures 5.12, 5.13 and 5.14. These plots show that saturation with  $\text{NAD}^+$  has occurred at about  $80 \mu\text{M}$   $\text{NAD}^+$  and the concentration of  $\text{NAD}^+$  which results in half maximum quenching of fluorescence is  $43 \mu\text{M}$  at  $20^\circ\text{C}$ ,  $27 \mu\text{M}$  at  $30^\circ\text{C}$  and  $20 \mu\text{M}$  at  $40^\circ\text{C}$ .  $1/\Delta F$  versus  $1/[L]$  plots are shown in Figures 5.15, 5.16 and 5.17 where the  $K_d$  values are calculated from the intersections on the X-axes. These  $K_d$  values are very similar to those estimated by equilibrium dialysis at the temperatures studied.

### 5.2.8 Circular dichroism

As outlined in section 5.2.5., equilibrium dialysis was carried out in phosphate/ $\text{NaCl}$  buffer containing  $1 \text{ M}$  urea. In order to determine whether this urea concentration had any effect upon the tertiary structure of the toxin, and therefore the nature of the  $\text{NAD}^+$ -binding site, the technique of circular dichroism was employed. This would illustrate any change in protein tertiary and secondary structure resulting from exposure to  $1 \text{ M}$  urea.

Circular dichroism spectra were obtained from pertussis toxin solutions in the

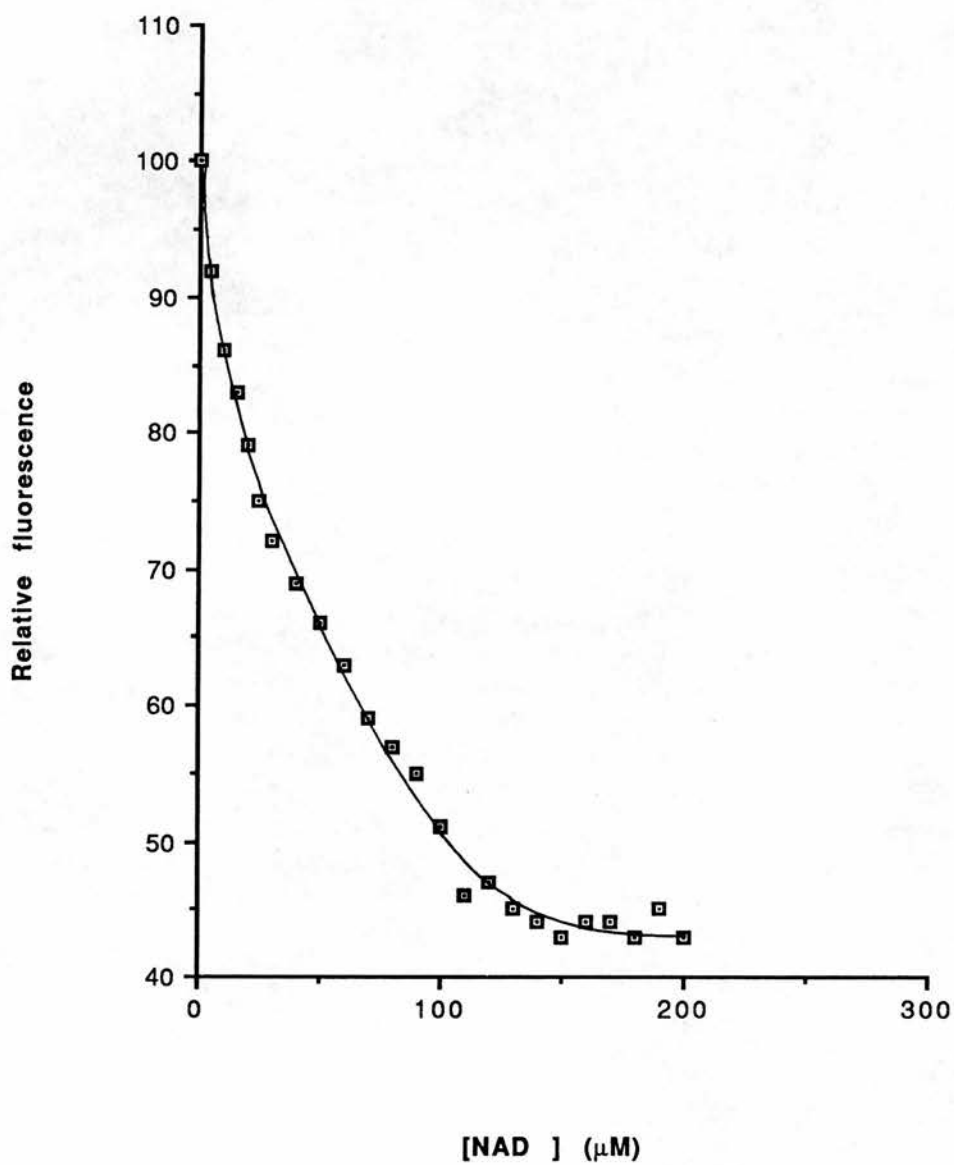




**FIGURE 5.12**

**FLUORESCENCE TITRATION OF PERTUSSIS TOXIN  
INTRINSIC FLUORESCENCE WITH NAD<sup>+</sup> AT 20°C.**

**K<sub>d</sub> = 43μM**



**FIGURE 5.13**

**FLUORESCENCE TITRATION OF PERTUSSIS TOXIN  
INTRINSIC FLUORESCENCE WITH NAD<sup>+</sup> AT 30°C.**

**K<sub>d</sub> = 28μM**

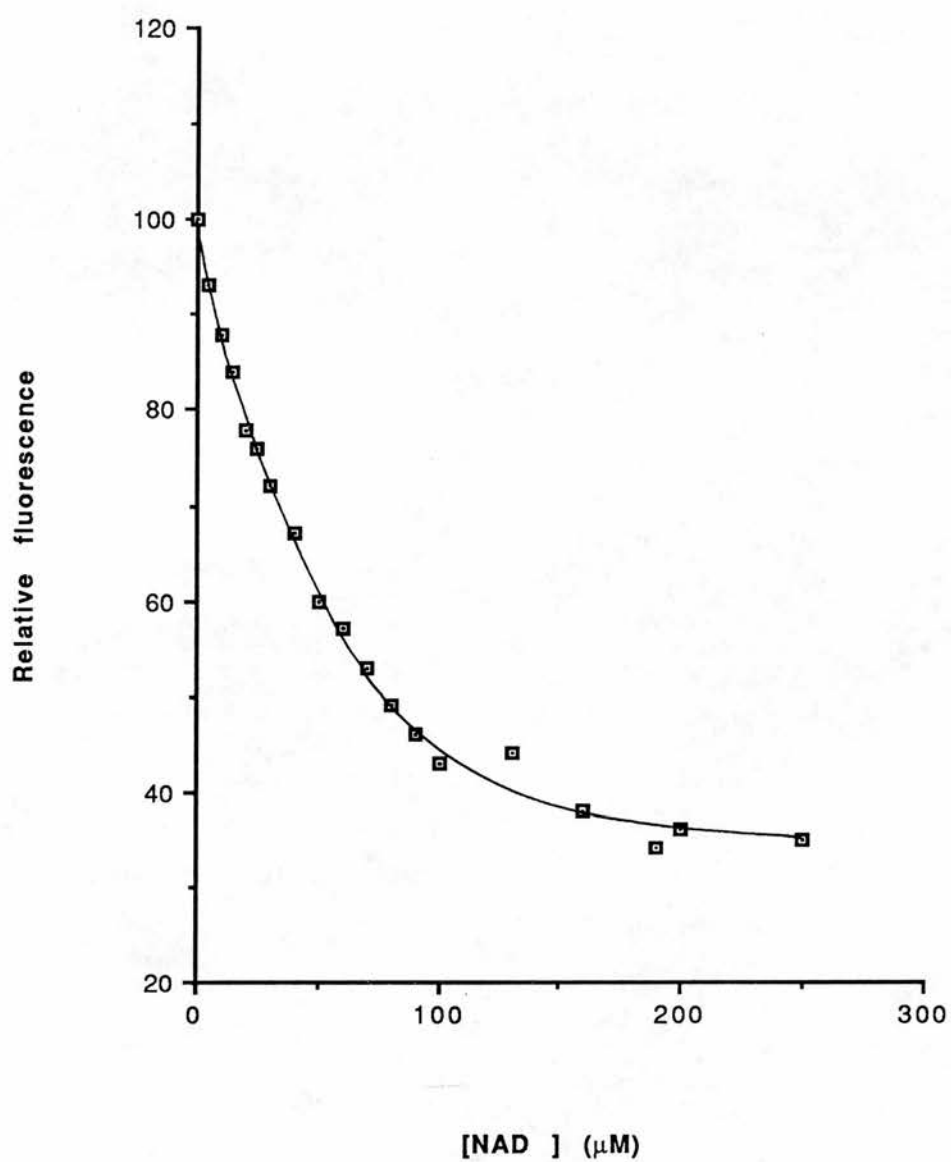


FIGURE 5.14

FLUORESCENCE TITRATION OF PERTUSSIS TOXIN  
INTRINSIC FLUORESCENCE WITH NAD<sup>+</sup> AT 40°C.

K<sub>d</sub> = 20 μM

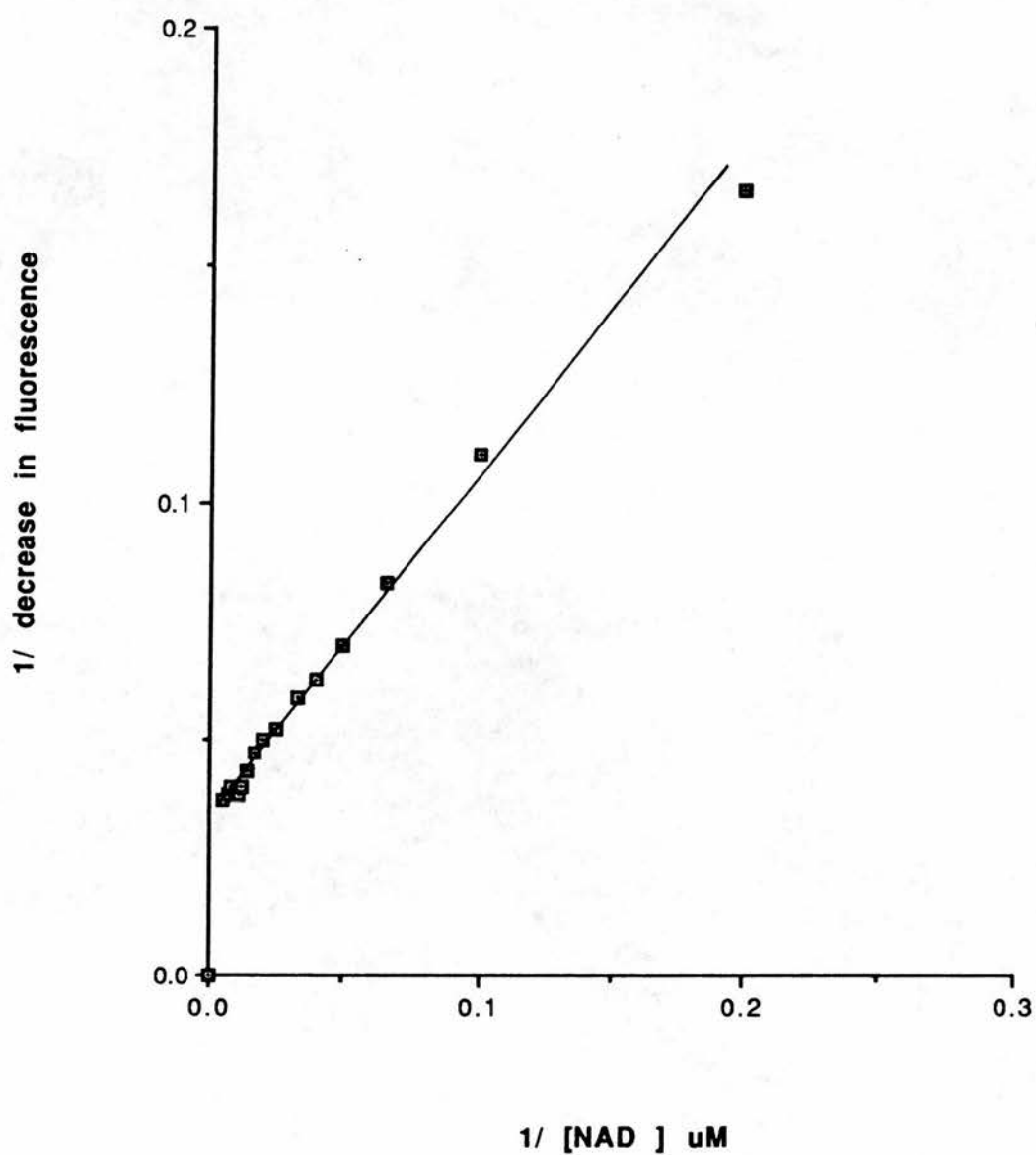
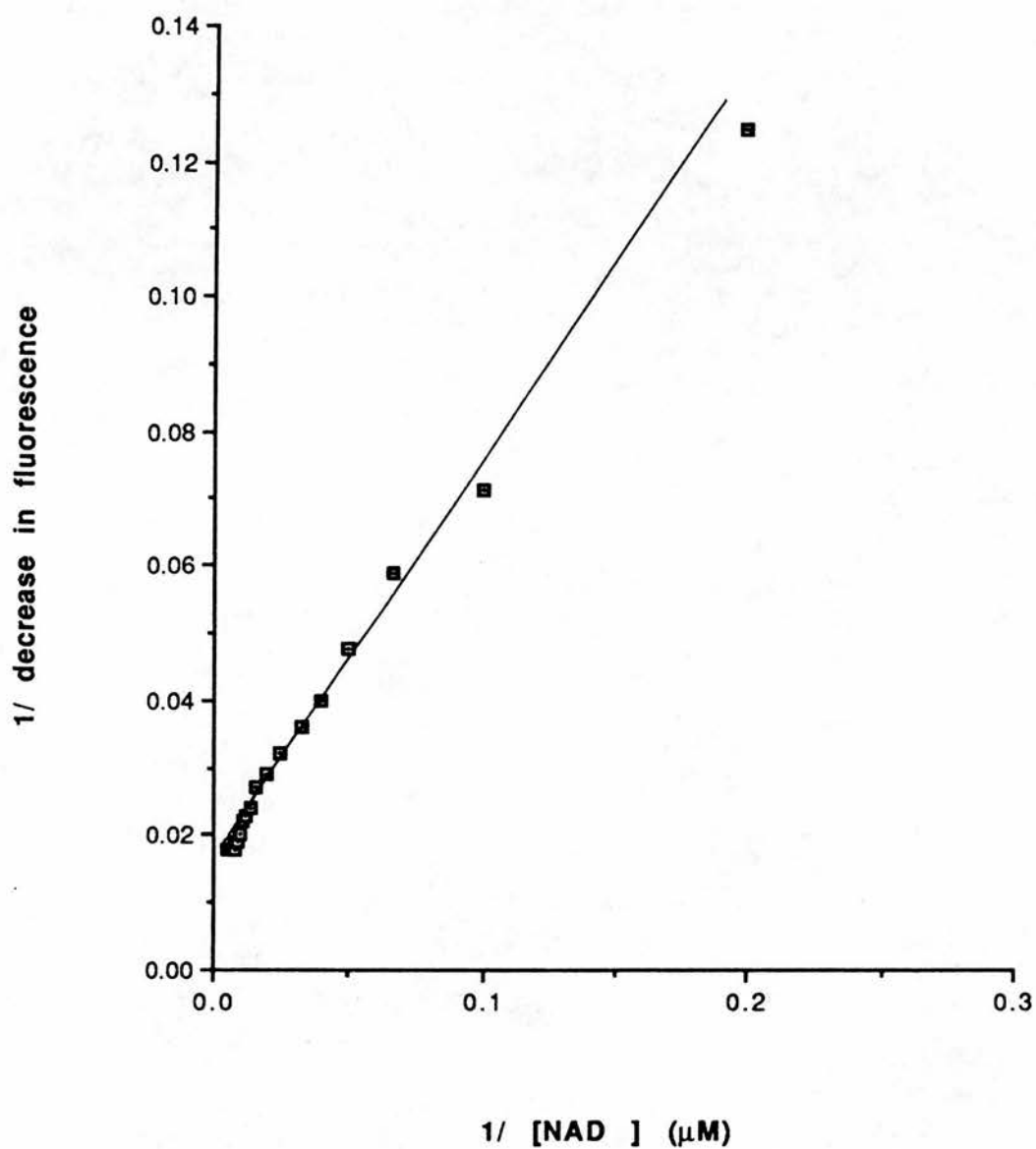


FIGURE 5.15

DOUBLE RECIPROCAL PLOT FOR FLUORESCENCE

TITRATION AT 40°C.

$K_d = 20\mu\text{M}$



**FIGURE 5.16**

**DOUBLE RECIPROCAL PLOT FOR FLUORESCENCE**

**TITRATION AT 30°C.**

**K<sub>d</sub> = 28μM**

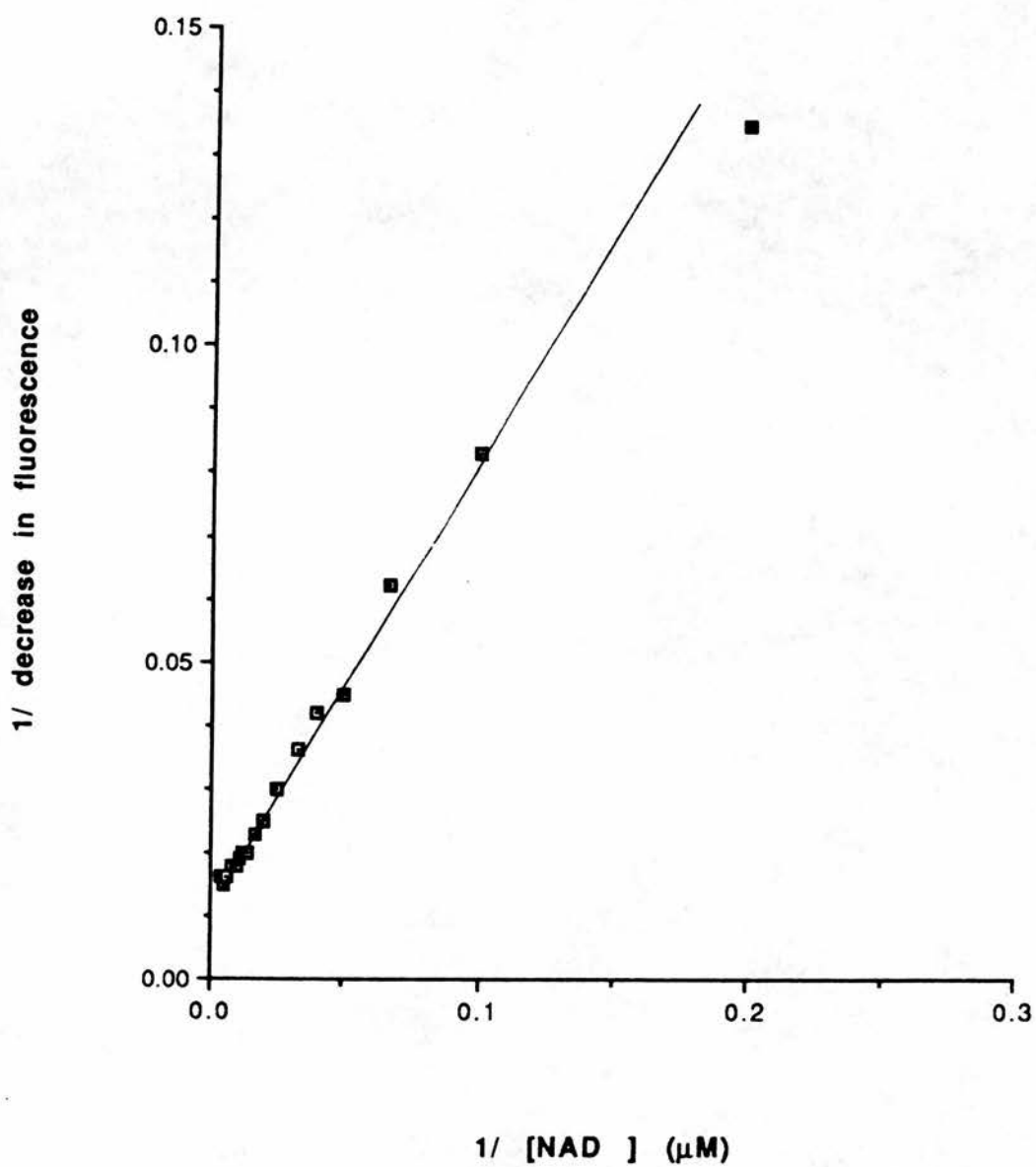


FIGURE 5.17

DOUBLE RECIPROCAL PLOT FOR FLUORESCENCE  
TITRATION AT 20°C.

$K_d = 40\mu\text{M}$

presence and absence of urea. Traces were from both the near ultra-violet (300 - 250 nm), Figure 5.18 and the far ultra-violet (200 - 250 nm), Figure 5.19 and 20. Figure 5.21 and Figure 5.22 show buffer blanks (10 mM potassium phosphate 0.1 M, NaCl, pH 7.5; 1 M urea buffer or 10 mM potassium phosphate, 0.1 M, NaCl, pH 7.2), which were subtracted from the spectra obtained from the toxin. Changes in tertiary structure is shown by near UV. Circular dichroism scans and far UV scans will show changes in secondary structure. The pathlength of the near UV cell was 1 cm and that of the far UV cell was 0.02 cm. Each scan took 5 minutes at a rate of 10 nm/minute. Each trace represents the average of four scans. Urea was shown to have negligible effect upon the tertiary structure of the protein within the error limits of the CD instrument. This is verified by the results gained by fluorescence which shows an identical  $K_d$  value for the binding of  $NAD^+$  to the toxin, confirming that the nature of the  $NAD^+$  binding pocket has not been altered. This was as expected as concentrations of greater than 2 M urea have been previously shown to be necessary in order to dissociate S-1 from the holotoxin (Rappuoli and Silvestri, 1987). From the CD traces we assumed that 1 M urea had no effect upon binding of  $NAD^+$  to pertussis toxin.

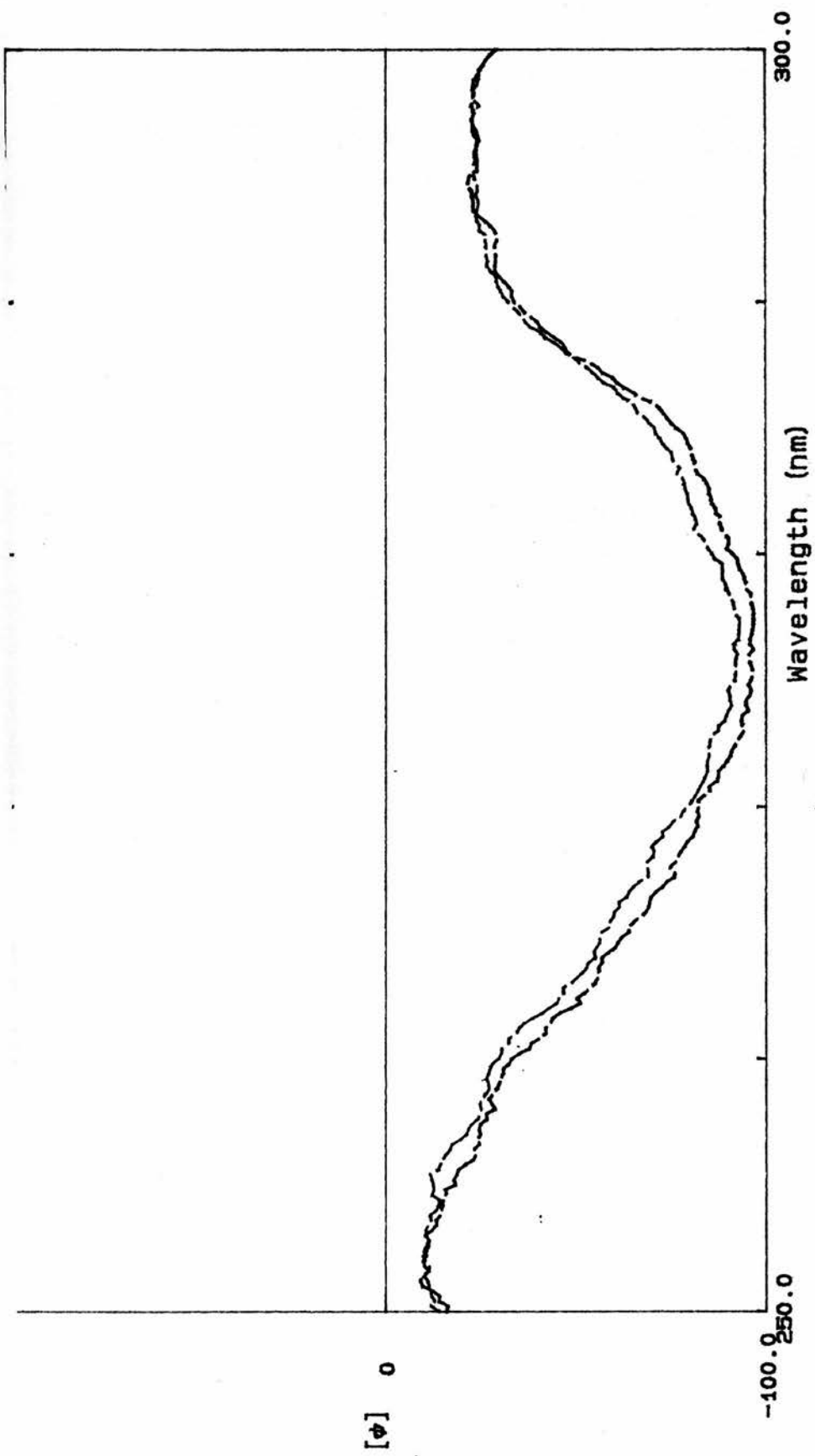
### 5.3. DISCUSSION

In this chapter the binding of  $NAD^+$  to pertussis toxin has been studied by two different methods, firstly by the direct method of equilibrium dialysis and secondly, indirectly by titration of the proteins intrinsic fluorescence with  $NAD^+$ . The  $K_d$  values derived using both techniques were in close agreement and correlated well with the  $K_m$  value calculated in chapter 4 for pertussis toxin  $NAD^+$ -glycohydrolase activity. It has also been shown that the number of binding sites for  $NAD^+$  per toxin molecule is one.

**FIGURE 5.18**

**FAR ULTRA-VIOLET CIRCULAR DICHROISM FOR  
PERTUSSIS TOXIN IN 10mM POTASSIUM PHOSPHATE, 0.1M  
NaCl, pH 7.2 BUFFER (mr 3pt 161) AND IN 10mM POTASSIUM  
PHOSPHATE 0.1M NaCl, 1M UREA, pH 7.2 BUFFER (mr 4pt  
161)**

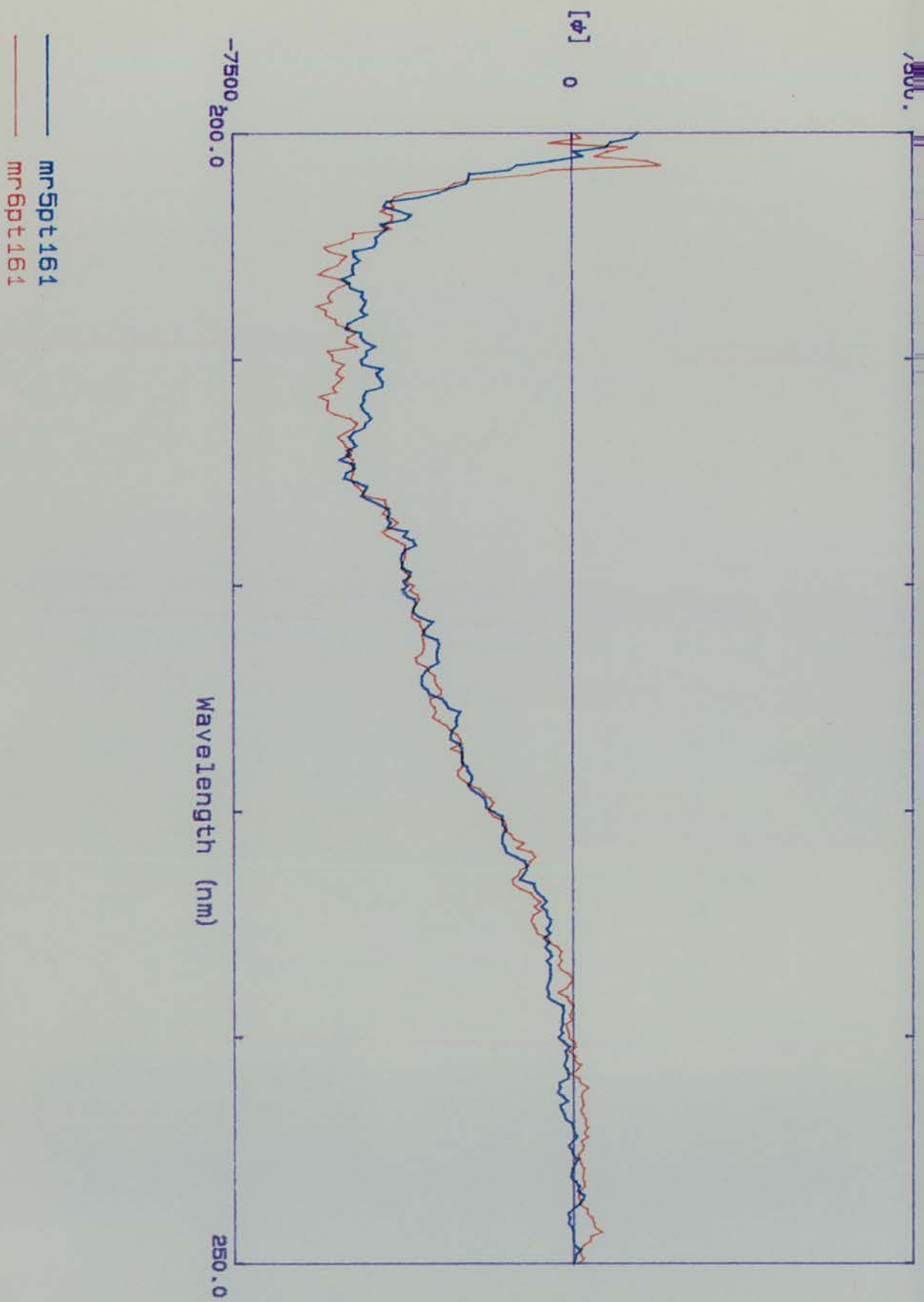




— mr3pt161  
- - - mr4pt161

**FIGURE 5.19**

**NEAR ULTRA-VIOLET CIRCULAR DICHROISM TRACE FOR  
PERTUSSIS TOXIN IN 10mM POTASSIUM PHOSPHATE, 0.1M  
NaCl, pH 7.2 BUFFER (mr 5pt 161) AND IN 10mM POTASSIUM  
PHOSPHATE 0.1M NaCl, 1M UREA, pH 7.2 BUFFER (mr 6pt  
161)**



**FIGURE 5.20**

**SUPERIMPOSITION OF FAR ULTRA-VIOLET CIRCULAR  
DICHROISM TRACES OF PERTUSSIS TOXIN IN 10mM  
POTASSIUM PHOSPHATE, 0.1M NaCl, pH 7.2 BUFFER AND  
IN 10mM POTASSIUM PHOSPHATE, 0.1M NaCl, 1M UREA pH  
7.2 BUFFER.**

**mr 5pt 161**

**mr 6pt 161            pertussis toxin in 10mM potassium phosphate,  
mr 7pt 161            0.1M NaCl, pH 7.2, buffer**

**mr 8pt 161**

**mr 9pt 161            pertussis toxin in 10mM potassium phosphate,  
mr 10pt 161          0.1M NaCl, 1M urea, pH 7.2, buffer**

7500.

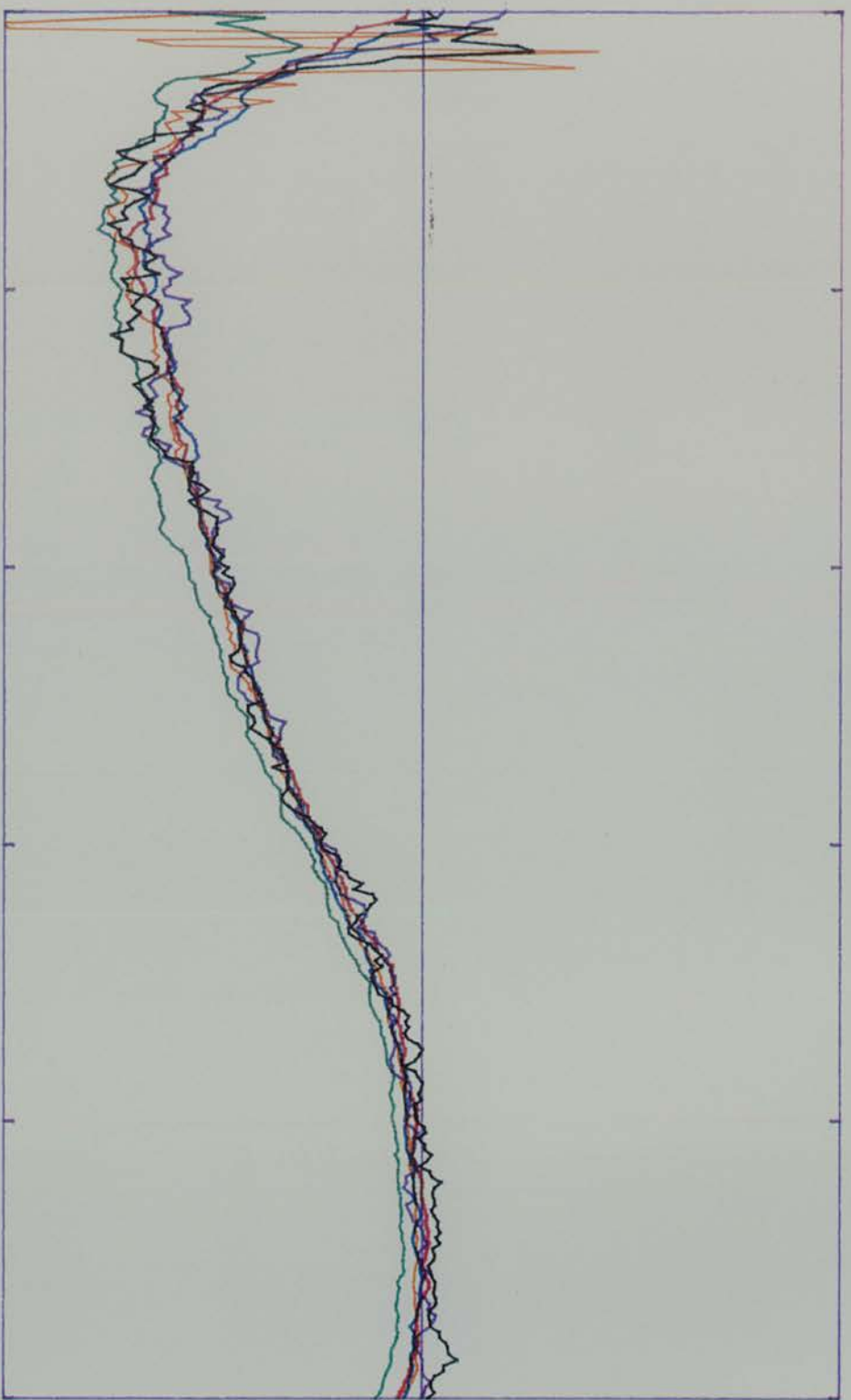
[ $\phi$ ] 0

-7500  
200.0

Wavelength (nm)

250.0

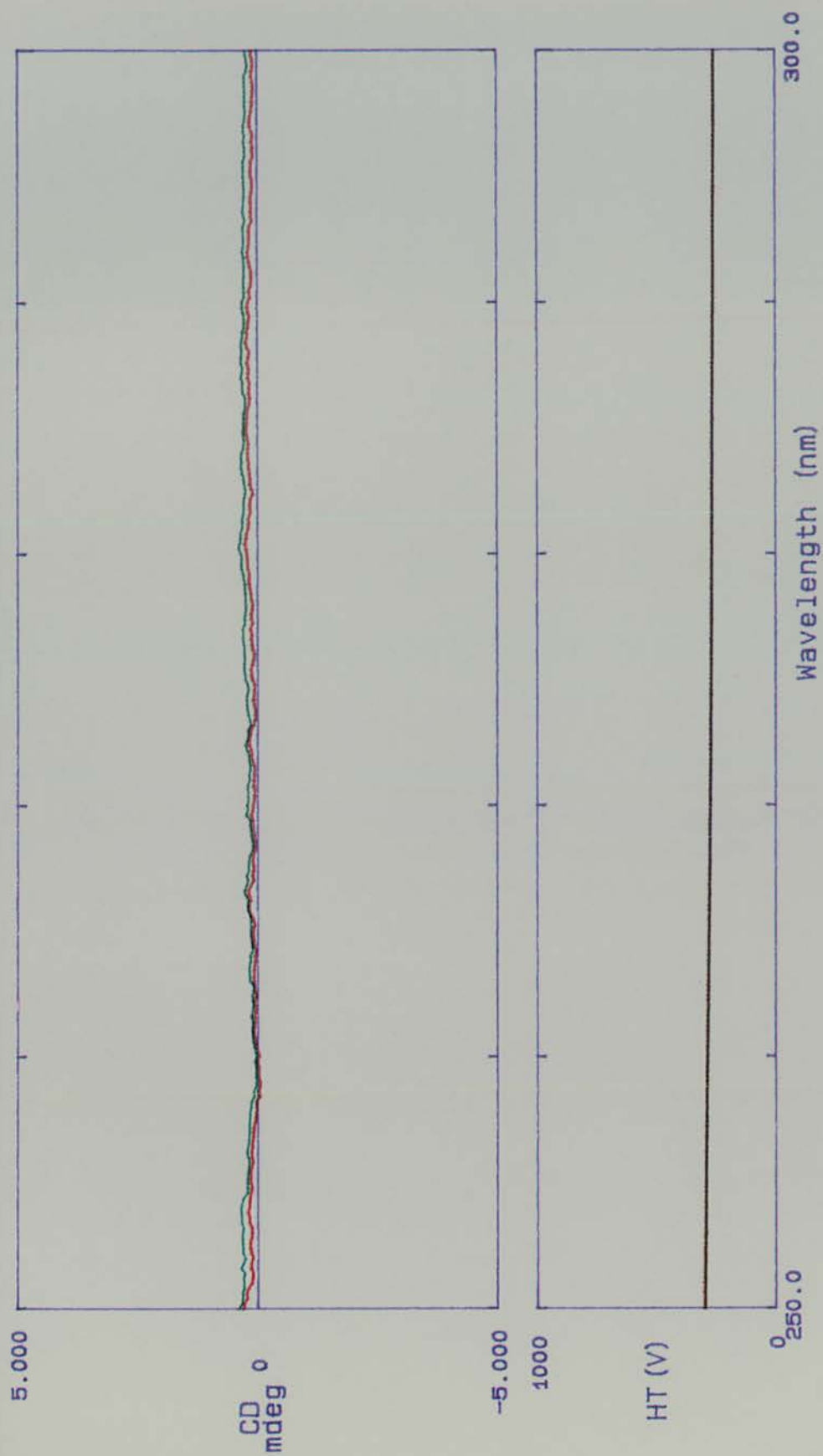
- mr5pt161
- mr6pt161
- mr7pt161
- mr8pt161
- mr9pt161
- mr10pt161



**FIGURE 5.21**

**BLANK CIRCULAR DICHROISM TRACE FOR BUFFERS IN  
THE NEAR ULTRA-VIOLET.**

- |                |  |
|----------------|--|
| <b>4 blank</b> | <b>10mM potassium phosphate, 0.1M NaCl, pH 7.2<br/>buffer</b>          |
| <b>3 blank</b> | <b>10mM potassium phosphate, 0.1M NaCl, 1M urea ph<br/>7.2 buffer.</b> |



— 4b1ank  
— 3b1ank

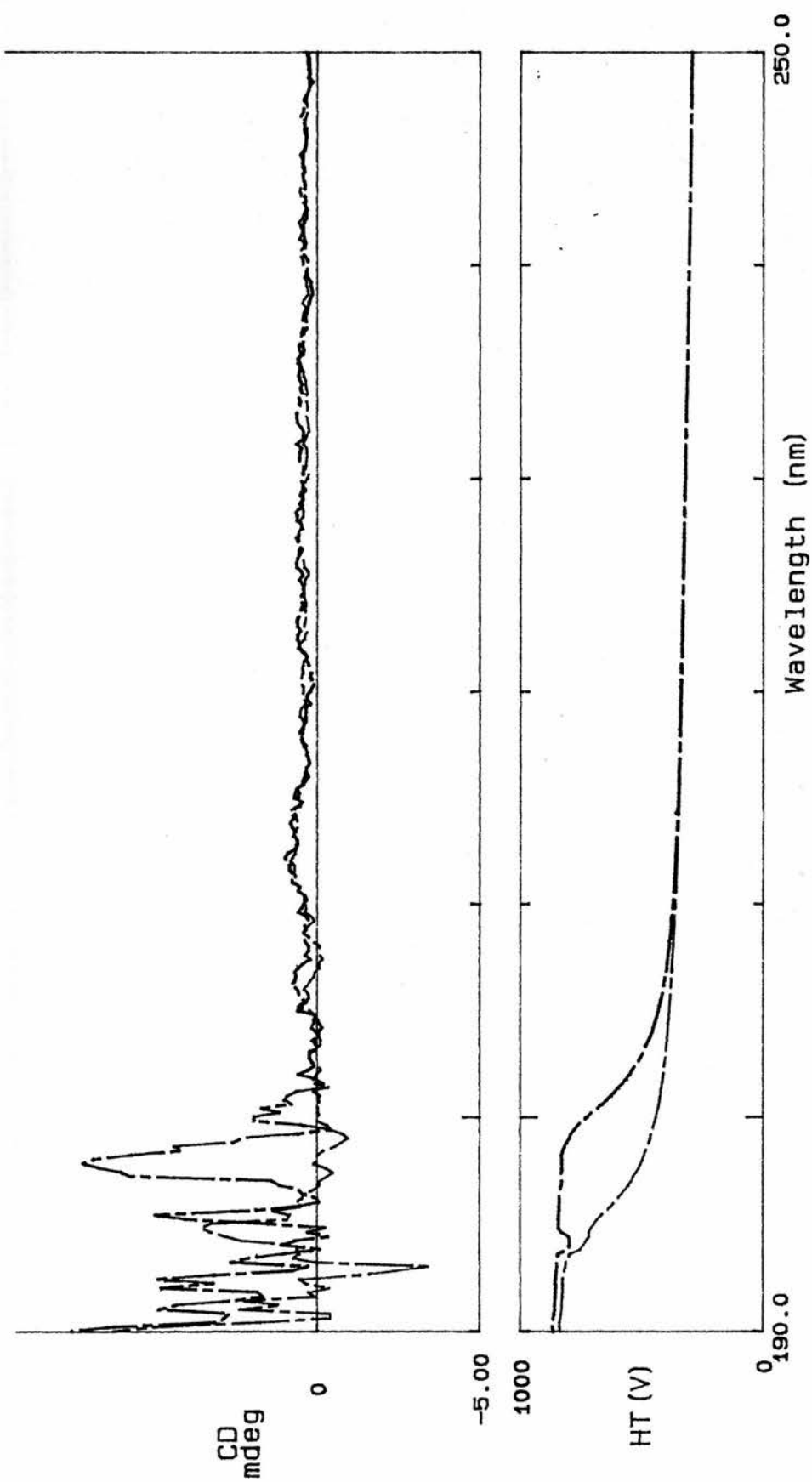
**FIGURE 5.22**

**BLANK CIRCULAR DICHROISM TRACE FOR BUFFERS IN  
THE FAR ULTRA-VIOLET.**

- |                |  |
|----------------|--|
| <b>5 blank</b> | <b>10mM potassium phosphate, 0.1M NaCl, pH 7.2<br/>buffer</b>          |
| <b>6 blank</b> | <b>10mM potassium phosphate, 0.1M NaCl, 1M urea ph<br/>7.2 buffer.</b> |

**THE TRACE BECOMES VERY NOISY AFTER 200nm, SO FAR  
ULTRA-VIOLET SCANS WERE ONLY TAKEN DOWN TO  
200nm.**





--- 5blank  
--- 6blank

### 5.3.1. Equilibrium dialysis

The first technique employed for binding studies was equilibrium dialysis. This is the simplest method of measuring protein-ligand binding in soluble systems. However, Kandel *et al* (1974) demonstrated that this method was not suitable for determining NAD<sup>+</sup> binding to diphtheria toxin. This was a consequence of the hydrolysis of NAD<sup>+</sup> by diphtheria toxin, which was sufficiently rapid to prevent the use of equilibrium dialysis to measure NAD<sup>+</sup> binding to this toxin. This was not the case for pertussis toxin as the NAD<sup>+</sup>ase activity was negligible under the conditions used and the NAD<sup>+</sup>-glycohydrolase activity is a very inefficient reaction ( $k_{cat}$  0.25 min<sup>-1</sup>) even when the toxin is fully activated. However, the negligible breakdown of NAD<sup>+</sup> is probably as a consequence of the lack of reducing agent within the dialysis mixture thus maintaining the inactivity of the protein. This is an interesting phenomenon as the binding results show that NAD<sup>+</sup> will bind to the toxin even if the protein is not in its active conformation, suggesting the existence of a ligand binding site and a separate catalytic site. The results also suggest that NAD<sup>+</sup> binds to the inactive form of the enzyme as well as it would to the active form as the  $K_d$  value calculated is very similar to the  $K_m$  value and the binding results exhibit saturation at levels of NAD<sup>+</sup> which were shown to be saturating on enzyme kinetic analysis.

Several  $K_d$  values were obtained for binding of NAD<sup>+</sup> to pertussis toxin, each at a different temperature, and from these values, a value of the enthalpy change on binding of NAD<sup>+</sup> to the toxin of 30 kJoules.mol<sup>-1</sup> derived.

The greatest source of error with equilibrium dialysis is the insolubility of pertussis toxin at the high concentrations of protein required for dialysis experiments. This was overcome by the incorporation of 1 M urea within the dialysis buffer, which enabled protein concentrations of greater than 1 mg/ml to be used. This urea concentration was shown to have negligible effect upon the tertiary structure of the

protein and as such would probably not cause any change in the nature of the toxins NAD<sup>+</sup>-binding site. This higher protein concentration enabled accurate and reproducible calculation of free-ligand concentration.

### 5.3.2. Calculation of constants

The results from equilibrium dialysis are shown graphically as plots of  $1/n$  versus  $1/[L]$ , a double reciprocal plot of moles of ligand bound per mole of protein ( $n$ ) versus free ligand concentration - a Hughes-Klotz plot in preference to the more familiar Scatchard plot (Scatchard, 1949). Nimmo *et al.*, (1977) reviewed a number of methods for analysing data obtained from experiments of similar design to those described in this chapter, and concluded that the regression of  $1/n$  versus  $1/[L]$  was preferred because it gave estimates that were accurate, precise and symmetrical in distribution. This plot is loosely equivalent to the Lineweaver-Burk plot of enzyme kinetics (Lineweaver and Burk, 1934) a plot which is not favoured by many workers, as outlined in chapter 4, because of the unequal distribution of errors it produces. It has been suggested that the differences in suitabilities of the double reciprocal plot between the two different situations is because in enzyme kinetics one of the variables, substrate concentration, is a fixed parameter and as such is free of error, whereas in equilibrium dialysis both of the derived variables,  $n$  and  $[L]$  are subject to experimental error which differs for each ligand concentration (Nimmo *et al.*, 1977).

### 5.3.3. Intrinsic fluorescence

Measurement of the changes in the intrinsic fluorescence of pertussis toxin provided another method of determining binding of ligand to protein. Tryptophan has been shown to fluoresce at 333nm when excited at 280 nm and it has also been shown using a model compound, indolyl ethyl-nicotinamide, that the fluorescence of the indolyl ring is completely quenched by its combination with nicotinamide. Kandel *et al.*, (1974) and Chung and Collier (1977), used this observation to determine NAD<sup>+</sup>

quenching of diphtheria toxin fragment A and *Pseudomonas aeruginosa* exotoxin A intrinsic fluorescence. This quenching in fluorescence is thought to result from formation of a charge transfer complex between the nicotinamide moiety of  $\text{NAD}^+$  and a tryptophan residue in each protein. Barbieri et al., (1989) and Cortina and Barbieri (1989) have shown that Trp 26 is important for the efficient binding of  $\text{NAD}^+$  to pertussis toxin suggesting that a similar charge transfer complex may exist within this protein on binding of substrate.

Quenching of ~ 50% of the toxins intrinsic fluorescence occurred on titration with  $\text{NAD}^+$  and  $K_d$  values of 43  $\mu\text{M}$  at 20°C, 28  $\mu\text{M}$  at 30°C and 20  $\mu\text{M}$  at 40°C calculated. These values agree well with those determined by equilibrium dialysis at the same temperatures, therefore confirming the results from this method.

#### 5.3.4. The nature of the $\text{NAD}^+$ -binding site

Equilibrium dialysis and fluorescence demonstrated that there is one  $\text{NAD}^+$ -binding site per toxin molecule.  $\text{NAD}^+$  binds with a high affinity to pertussis toxin, the  $K_d$  of 25  $\mu\text{M}$  being of the same order of magnitude to those of diphtheria toxin (Kandel et al., 1974) and *Pseudomonas aeruginosa* exotoxin A (Chung and Collier, 1977). Both prokaryote and eukaryote ADP-ribosyltransferases (Moss et al., 1983; Gill, 1978) have  $K_d$  values for  $\text{NAD}^+$  within the micromolar range, which would be as expected as the normal cellular concentration of  $\text{NAD}^+$  is 70 - 100  $\mu\text{M}$  (Gill, 1975).

It has been shown with cholera toxin, diphtheria toxin and exotoxin A, using  $\text{NAD}^+$  analogues, that  $\text{NAD}^+$  binding involves recognition of the entire molecule with the nicotinamide ring being especially important. A  $\beta\alpha\beta\alpha$  secondary structure has been predicted for cholera, diphtheria and *E. coli*. LT toxins (Duffy and Lai, 1986; Yamamoto et al., 1984). This suggested that a Rossman fold of alternating  $\alpha$ -helix and

$\beta$ -sheet structure may make up the  $\text{NAD}^+$ -binding sites of these proteins. This structure represents the  $\text{NAD}^+$ -binding sites of lactate dehydrogenase (Rossman *et al.*, 1974) and other dehydrogenases. However, this predicted structure was disregarded after the X-ray structure of exotoxin A found no such structure within this protein (Allured *et al.*, 1986) demonstrating that this protein did not contain a Rossman-fold type structure. Whether this is the case with pertussis toxin or not also requires X-ray crystallographic determination of its structure.

Binding of  $\text{NAD}^+$  to pertussis toxin results in the enzyme catalysed hydrolysis of  $\text{NAD}^+$  producing nicotinamide and ADP-ribose. The nicotinamide-ribose linkage must therefore be accessible to nucleophilic substitution by water. ADP-ribose is subsequently accepted by Gi suggesting that there may be an appropriately placed nucleophilic group on this protein. Tait and Nassau (1984), have suggested that the active site of cholera toxin may comprise a hydrophobic pocket, which is responsible for the efficient binding of substrate and a hydrophilic region associated with the site of ADP-ribosylation. If this is the case with pertussis toxin then tyrtophan 26, along with certain other residues within S-1, would constitute the hydrophobic binding pocket of the protein. The SH-group on Gi which accepts the ADP-ribose reaction product must also be recognised by the toxin, by the same mechanism as cysteine and the other sulphydryl reagents are recognised.

More recently Locht *et al.*, (1990) have suggested that Cys 41 in S-1 is located at the  $\text{NAD}^+$ -binding site in a position close to at least one of the phosphates of  $\text{NAD}^+$ . The evidence for this was obtained from site-directed mutagenesis of Cys 41 and kinetic analysis of the mutant proteins. Cys 41 was replaced with either serine, glycine, proline or asparagine and finally with glutamate or aspartate. Replacement with serine or glycine failed to abolish enzyme activity indicating that the sulphydryl group of Cys 41 is not essential for enzymic activity. No dramatic loss of activity was reported following replacement of Cys 41 with proline or asparagine. This indicated that the polarity of the side group at this location was not important, proline being non-polar and asparagine being polar. These results really suggested that Cys 41 did not play a role in

catalysis and that it was in fact either close to or within the  $\text{NAD}^+$ -binding site. Finally the introduction of a negative charge at position 41 abolished the  $\text{NAD}^+$ -glycohydrolase activity to 2% of the wild-type value. This dramatic effect suggested an electrostatic repulsion between the negative charge within the protein and a negative charge with the  $\text{NAD}^+$  molecule.

Cys 41 is located within the amino-terminal half of S-1 and close to Trp 26. Both of these residues are located between the two regions of high homology to cholera toxin and *E. coli*. heat labile toxin A-subunits. Trp 26 and Cys 41 may therefore define the  $\text{NAD}^+$ -binding site with the aromatic ring of Trp 26 interacting with the nicotinamide ring of  $\text{NAD}^+$ . Further evidence for this theory can be gained from the quenching of fluorescence data as this further implicates Trp 26 at the  $\text{NAD}^+$ -binding site.

Locht *et al* (1990) proposed the model for the functional structure of pertussis toxin S-1 subunit shown in Figure 5.23. As shown this group proposes that the amino terminal of S-1, which includes the two sequences of homology with cholera and L.T. toxins as well as Cys 41 and Trp 26 may constitute the  $\text{NAD}^+$ -binding site. The carboxy terminal and the middle portions may be involved in G-protein binding and finally Glu 129 may be important in catalysing the transfer of the ADP-ribose moiety from  $\text{NAD}^+$  to the acceptor protein.

This is all very well, but the results from site-directed mutagenesis do not necessarily prove that either Cys 41 or Trp 26 make up the  $\text{NAD}^+$ -binding domain. The effects on the kinetics of S-1 after mutagenesis could result from a conformational change induced by the mutation. The fact that  $\text{NAD}^+$  quenches the intrinsic fluorescence of S-1 does add further evidence to the participation of Trp 26 within the  $\text{NAD}^+$ -binding site. However, the fluorescence quench does not necessarily result as a consequence of the interaction of  $\text{NAD}^+$  with this tryptophan but may perhaps form its interaction of another within the amino-terminal of S-1. For these questions to be

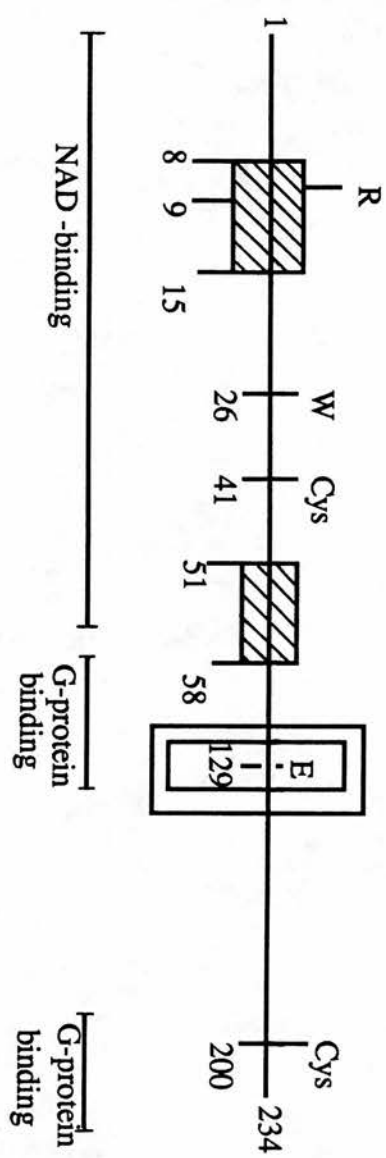
answered in full X-ray crystallographic analysis of the toxin must be undertaken. Only then will a positive three-dimensional structure of the toxin and its constituent domains be obtained.

**FIGURE 5.23**

**PROPOSED MODEL FOR THE FUNCTIONAL STRUCTURE OF  
PERTUSSIS TOXIN S-1.**

**ADAPTED FROM LOCHT ET AL (1989)**





## CHAPTER 6

### CROSS-LINKING OF PERTUSSIS TOXIN SUBUNITS

#### 6.1. INTRODUCTION

The structure of pertussis toxin is discussed in section 1.2.3., however, as an introduction to this chapter the model proposed to date is summarised. The overall structure of this protein is the most complicated of the bacterial toxins discovered to date, consisting of five different subunits, termed S-1 through to S-5 on the basis of their decreasing molecular masses, within the mature protein. Its molecular mass has been the subject of much controversy in recent years with reported values ranging from 100kDa (Morse and Morse, 1976) up to 170kDa (Cowell *et al.*, 1982). Tamura *et al.*, (1982) suggested that the molar ratios of subunits S-1, S-2, S-3, S-4 and S-5 within the holotoxin was 1:1:1:2:1. This proposed stoichiometry was based upon evidence from the densitometric scanning of Coomassie blue stained polyacrylamide gels. This was confirmed by Sekura *et al.* (1983) by the same method. However, in calculating this stoichiometry both groups made the assumption that each subunit had an equal capacity for Coomassie blue binding. This is very unlikely as dye binding is a property of the overall charge of a protein, with more positively charged proteins binding more dye, thus each of the toxin subunits should not bind dye at the same capacity.

Rappuoli and Nicosia (1987), demonstrated that in 2 M urea S-1 is dissociated from the B-oligomer complex. Further exposure of the B-oligomer to 5 M urea frees S-5 from the remaining subunits leaving dimers, D1 of S-2 and S-4 and D2 of S-3 and S-4. Finally exposure to 8 M urea causes all five subunits to dissociate. These subunit associations are further evidence for the existence of two dimers within the holotoxin and the 1:1:1:2:1 stoichiometry.

The final piece of evidence for this model was derived from the nucleotide sequence of the S-4 signal peptide. This leader sequence is forty two amino acids long, being twice as long as that for any of the other subunits, and it has a high amino terminal positive charge (Locht *et al.*, 1986; Nicosia *et al.*, 1986). Since the length of

this peptide and the magnitude of its N-terminal positive charge are both important for the efficient production of secreted proteins, it has been proposed that this signal peptide may lead to the production of two S-4 subunits per toxin molecule.

All of the above evidence points to the existence of two molecules of S-4 within the holotoxin. More recently Yamakawa *et al.*, (1990) have isolated pertussis toxin by HPLC, and, on the basis of UV absorption again proposed the 1:1:1:2:1 stoichiometry. In this chapter I have discussed an alternative approach in the investigation of the toxin's proposed stoichiometry and into the geometry of the toxin subunits in space using the methods of reversible cross-linking, two-dimensional gel electrophoresis and Western blotting.

## 6.2. Cross-linking

Cross-linking involves the use of bifunctional chemical reagents which form a number of intermolecular dimers, each of which contain a pair of neighbouring subunits. As each subunit can form a linkage with one or more of its neighbours, analysis of the subunits involved in each of the dimers should help to construct a map showing the relative spatial location of each of the subunits within the assembled toxin. This process is however further complicated as aggregates of more than two subunits can be formed which, in a complicated system, can be difficult to analyse.

Bifunctional cross-linking reagents are used to analyse the tertiary structure of proteins. These reagents modify amino acid side chains and are classified on several criteria:

- (a) the chemical specificity of the cross-linking reagent
- (b) the length of the reagent
- (c) whether the reagent cross-links the same amino acids (homobifunctional) or different amino acids (heterobifunctional)
- (d) whether the groups react chemically or photochemically
- or (e) whether the reagents contain a cleavable internal bond.

Most of the bifunctional reagents modify lysine or cysteine residues. Cleavable

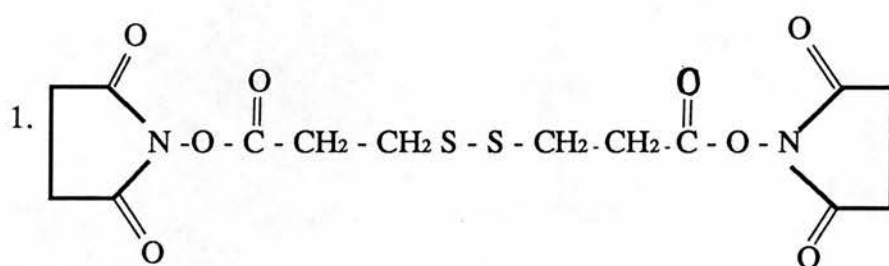
reagents dithiobis (succinimidyl propionate), DSP and disuccinimidyl tartarate, DST, were used to investigate the tertiary structure of pertussis toxin. Both of these reagents are homobifunctional, being specific for primary or secondary aliphatic amines (Tesser *et al*, 1975; Zarling *et al*, 1980) or N-terminal amines. The structures of both DSP and DST are shown in Figure 6.1. Both reagents are cleavable. DSP contains an internal disulphide bond and is cleavable with reducing reagents, DST is cleavable with periodate. This cleavable linkage helps in the identification of the subunits involved in each of these cross-linkages as cleavage of the reducible bonds regenerate the original monomeric subunits involved in each of the aggregates. This is very important for pertussis toxin work, as, being such a complicated protein many dimers and larger aggregates will be formed between the toxin subunits and analysis of their composition will require this two-step process.

The simplest method of determining which subunits are involved in each aggregate is by two-dimensional SDS polyacrylamide gel electrophoresis. This allows separation of the cross-linked dimers, and aggregates of three or more of the subunits, in the first dimension and then separation of the regenerated monomeric subunits in the second dimension following cleavage of the cross-link bridge.

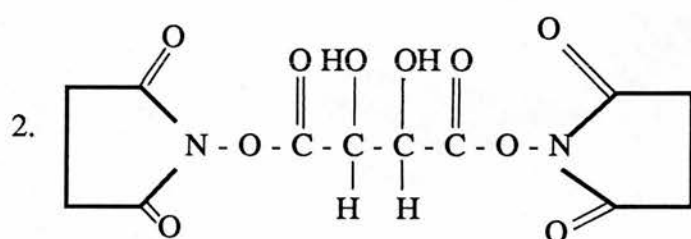
### 6.3. Two dimensional SDS polyacrylamide gel electrophoresis

The general two-dimensional SDS polyacrylamide gel electrophoresis system employed for analysis of cross-linked pertussis toxin subunits is outlined in section 2.2.4.2. The cross-linking reagents DSP or DST were dissolved in a minimal amount of DMSO, as they have limited water solubility, with water being added to give final linker concentrations of 100 mM and 10 mM. The reagent was added to a 1 mg/ml toxin solution and the reaction allowed to proceed in the cross-linker/solvent emulsion. The reaction is favoured by alkaline pH which maintains the amine group in an unprotonated state. Experiments were performed in phosphate buffered saline at pH 8.3 and cross-linking reagent was added to the toxin solutions to a final concentration of 1 - 10 mM reagent. The emulsion was incubated at room temperature for 4 hours.

The cross-linked reaction products were boiled in 0.1% SDS and separated on a



DSP  
Dithiobis(succinimidyl propionate)  
12A



DST  
Disuccinimidyl tartarate  
6.4A

**FIGURE 6.1**

**THE STRUCTURES OF THE CROSS-LINKING REAGENTS  
USED FOR STRUCTURAL STUDIES OF PERTUSSIS TOXIN  
SUBUNITS.**

first dimension 5% (w/v) - 20% (w/v) or 10% (w/v) - 20% (w/v) acrylamide linear gradient gels under denaturing conditions. Strips from the first dimension were then excised, the cross-linkage reduced, and the monomeric subunits resolved on a second dimension 17.5% (w/v) acrylamide gel.

## **6.4. RESULTS**

### **6.4.1 Two dimensional SDS polyacrylamide gels**

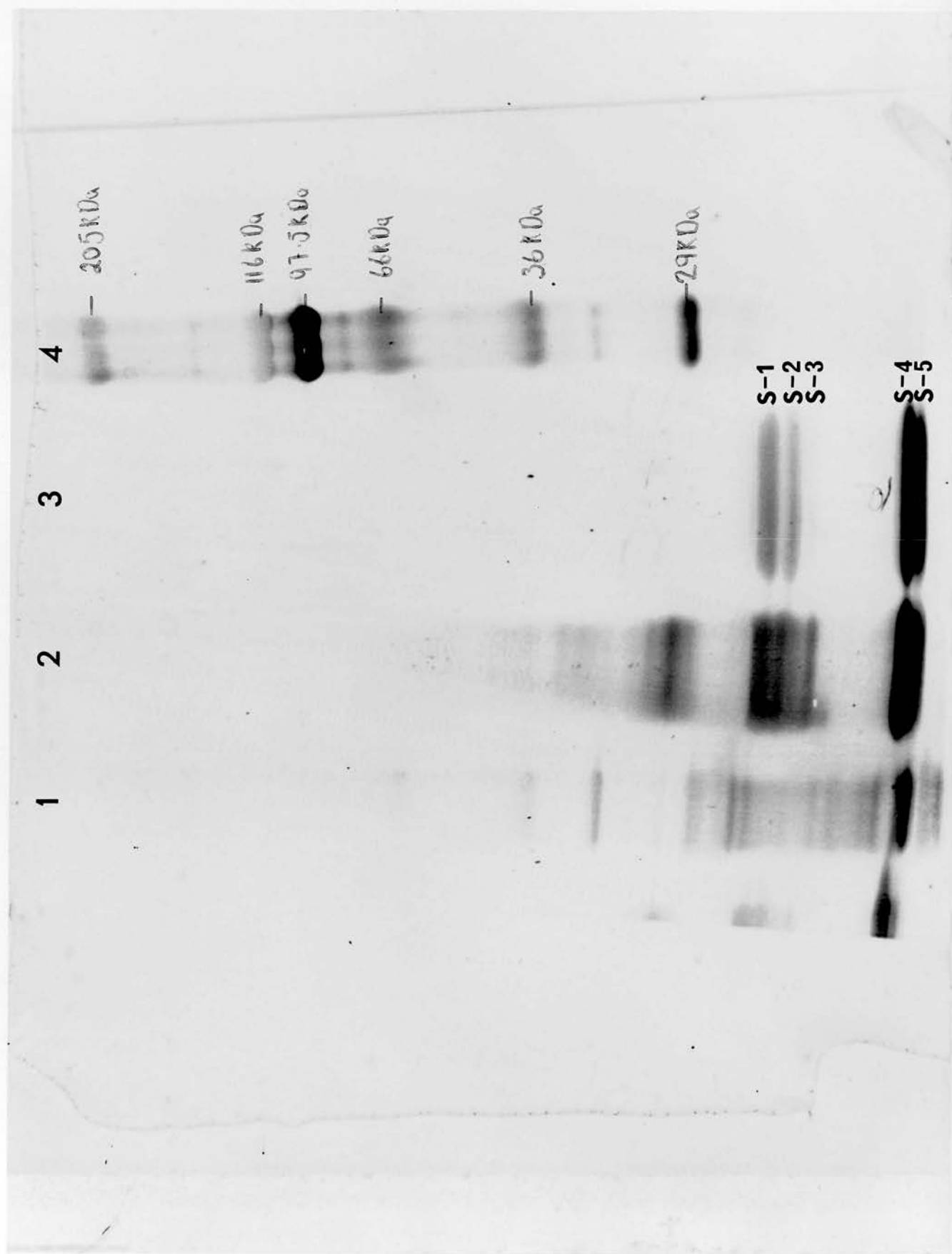
Figure 6.2 shows a 5% - 20% (w/v) acrylamide gradient first dimension SDS polyacrylamide gel of DSP cross-linked subunits. The molecular masses of the cross-linked species formed were determined by comparison with standard proteins of known mass and by comparison with the molecular mass of individual pertussis toxin subunits. As the molecular masses of the toxin subunits are known to great accuracy these were thought to be acceptable molecular mass standards. The mass of the cross-linked species calculated from this gradient are shown in Table 6.1. However this gradient does not separate the lower molecular mass species very well and a 10% - 20% (w/v) acrylamide first dimension gel was next employed. Figures 6.3 and 6.4 show the separation of both DSP and DST cross-linked species obtained from this gradient. Again, the molecular masses of these cross-linked aggregates were determined and are shown in Table 6.1. The masses of the aggregates obtained with the DSP cross-linker suggests that S-1 has formed dimers with both subunits S-2 and S-3 and that S-4 has formed aggregates with S-2 and S-3.

The shorter, DST cross-linker, shows a different pattern of dimer formation, the calculated masses again being shown in Table 6.1.

**FIGURE 6.2**

**5% - 20% (w/v) ACRYLAMIDE SDS POLYACRYLAMIDE GEL  
OF PERTUSSIS TOXIN AND CROSS-LINKED PERTUSSIS  
TOXIN SUBUNITS.**

- |               |   |
|---------------|---|
| <b>LANE 1</b> | <b>low molecular weight markers</b>     |
| <b>LANE 2</b> | <b>10mM DSP cross-linker incubation</b> |
| <b>LANE 3</b> | <b>Pertussis toxin standard</b>         |
| <b>LANE 4</b> | <b>high molecular weight markers</b>    |





**FIGURE 6.3**

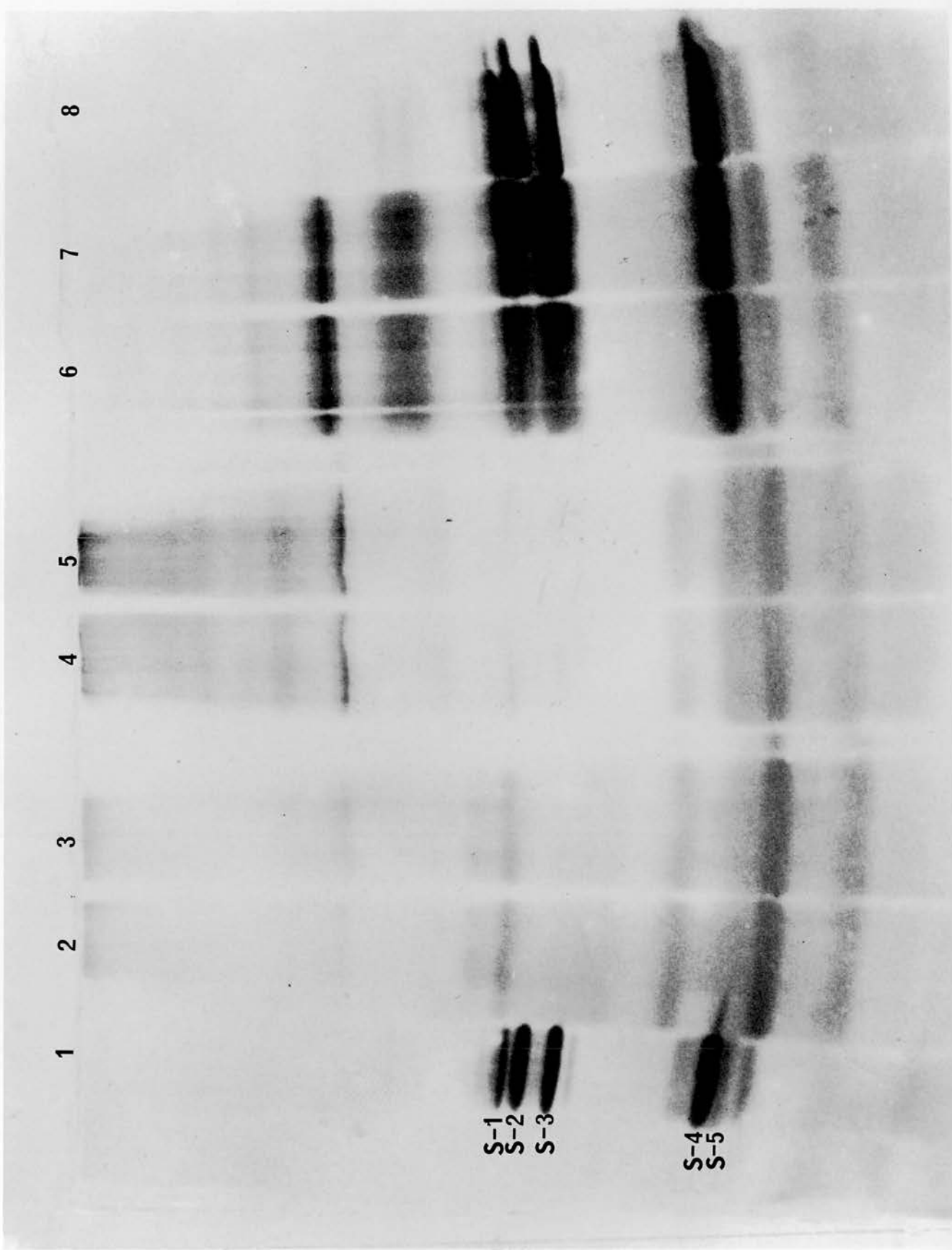
**10% - 20% (w/v) ACRYLAMIDE SDS POLYACRYLAMIDE GEL  
SHOWING SEPARATION OF CROSS-LINKED AGGREGATES.**

<b>LANE 1</b>	<b>Pertussis toxin standard</b>
<b>LANE 2</b>	<b>1mM DST in final reaction mixture</b>
<b>LANE 3</b>	<b>1mM DST in final reaction mixture</b>
<b>LANE 4</b>	<b>10mM DST in final reaction mixture</b>
<b>LANE 5</b>	<b>10mM DST in final reaction mixture</b>
<b>LANE 6</b>	<b>10mM DSP in final reaction mixture</b>
<b>LANE 7</b>	<b>10mM DSP in final reaction mixture</b>
<b>LANE 8</b>	<b>Pertussis toxin standard</b>

1 2 3 4 5 6 7 8

S-1  
S-2  
S-3

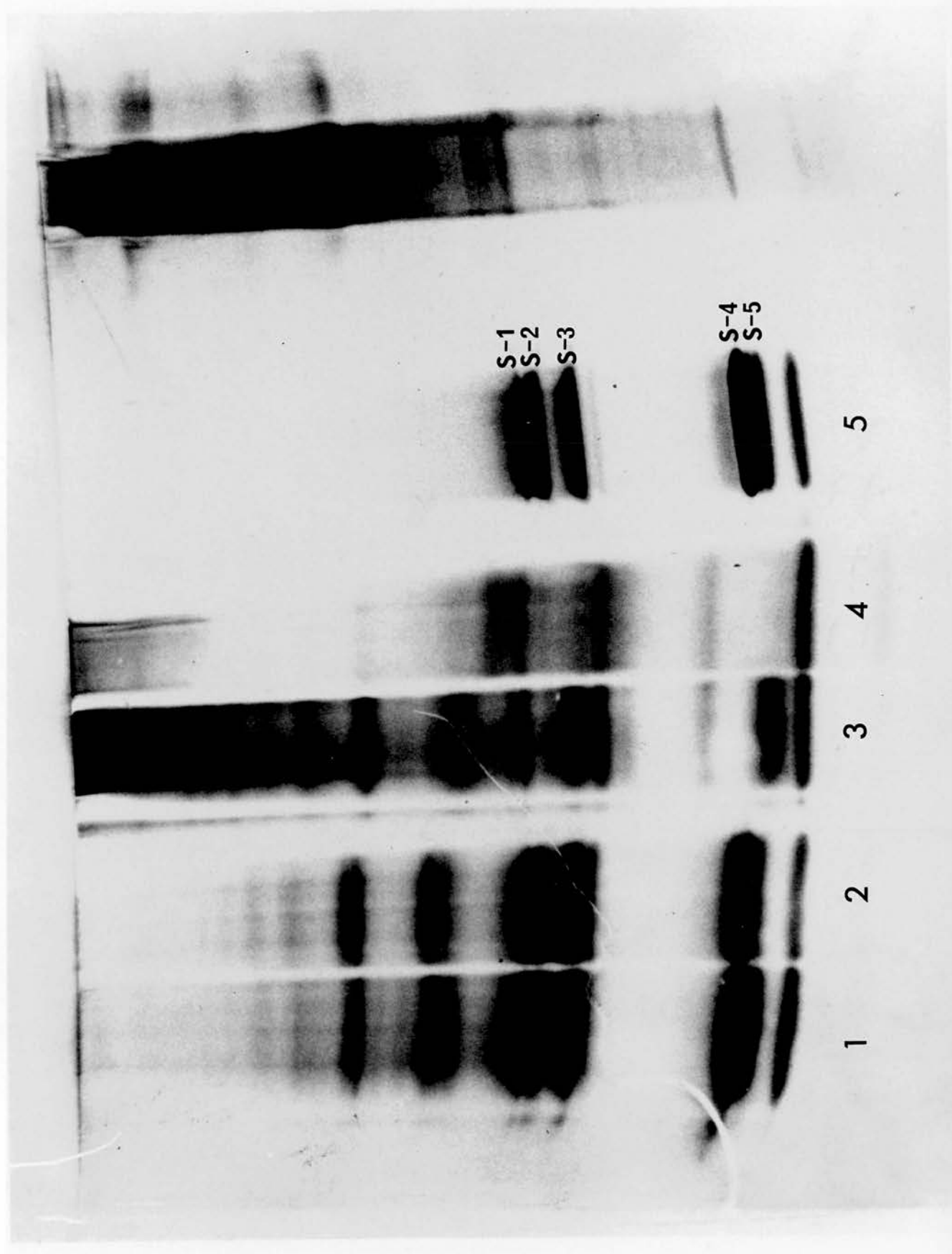
S-4  
S-5



**FIGURE 6.4**

**FIRST DIMENSION 10% - 20% (w/v) ACRYLAMIDE SDS  
POLYACRYLAMIDE GEL OF CROSS-LINKED SPECIES.**

<b>LANE 1</b>	<b>10mM DSP in final reaction mixture</b>
<b>LANE 2</b>	<b>1mM DSP in final reaction mixture</b>
<b>LANE 3</b>	<b>10mM DST in final reaction mixture</b>
<b>LANE 4</b>	<b>1mM DST in final reaction mixture</b>
<b>LANE 5</b>	<b>Pertussis toxin standard</b>
<b>LANE 6</b>	<b>high molecular weight markers</b>



**TABLE 6.1**                      **Estimated molecular masses of the cross-linked aggregates from the first-dimension gradient SDS polyacrylamide gel**

5% - 20% gel	10% - 20% gel	10% - 20% gel
DSP cross-linker	DSP cross-linker	DST cross-linker
48kDa	58kDa	62.5 kDa
44kDa	55kDa	60kDa
42kDa	51kDa	58kDa
34kDa	48.5kDa	55kDa
32kDa	34.2kDa	44kDa
	33.3kDa	32kDa

The calculated mass of each of the aggregates gives an idea of which subunits are involved in each of them, but because of the large number of possible dimers and aggregates of two or more subunits which could be formed, it is impossible to positively determine the composition of each. This is further complicated as the molecular mass of S-2 is almost identical to that of S-3 and they are both very close to the mass of S-1, with the mass of S-4 also being very similar to that of S-5. Thus the combined mass of two or more of these subunits, in many different combinations of the individual monomers, would result in an overall mass which could correspond to several individual combinations. This being the case a second dimension electrophoresis is essential to confirm which of the subunits are involved in each of the cross-linked combinations. Regeneration of the monomeric subunits is the first step in this two dimensional system, with the individual subunits being exposed on the second dimension SDS polyacrylamide gel.

Several forms of second dimension were attempted. Firstly, a strip was cut from the first dimension gel and soaked in a reducing buffer for 2 hours. This buffer

was 20 mM Tris, pH 6.7 containing 3 mM DTT (Brennen *et al* 1985), the reducing reagent presumably cleaving the internal disulphide bond of DSP. The strip was then turned through 90° and fixed to the top of a second dimension 17.5% (w/v) acrylamide separating gel for electrophoresis in this dimension (Zarling *et al*, 1980; Takada *et al*, 1984). None of the cross-linked species were dissociated into subunits by this method and each of the aggregates lies on the diagonal. Figure 6.5 shows a typical second dimension gel obtained from this method.

Reduction within the gel matrix itself was the technique next tried. 5 mM dithiothreitol was incorporated firstly within both the stacking and separating gel matrices and secondly within the stacking gel alone. Neither of these methods produced results which could confirm the predictions from the first dimension. Incorporation of thiol within both gel matrices produced gels which could not be interpreted. This was probably due to the presence of reducing reagent within the separating gel matrix as it had been previously demonstrated (Sekura *et al*, 1983) that pertussis toxin subunits separate differently under reducing conditions. Sekura *et al*, (1983) demonstrated that under reducing conditions the electrophoretic mobilities of the S-4 and S-5 subunits were reversed and that S-2 and S-3 co-migrate, making the identification of the subunits even more difficult. With this in mind the second attempt at reducing the cross-linkages within the gel matrix was carried out, but this time dithiothreitol was incorporated within the stacking gel matrix alone. The proteins from the first dimension strip were run into this stacking gel and once the aggregates were within the matrix the current was switched off for a 2 hour period. The cross-linker would presumably be cleaved during this time and once the current was reapplied, the regenerated monomeric subunits would be separated within the 17.5% (w/v) acrylamide resolving gel. This method was the most successful of the two dimensional systems employed. Figure 6.6 shows a typical gel obtained from this technique. Some of the cross-links have certainly been reduced within the second dimension, and it is possible to identify the subunits involved within the proposed S-1 and S-2 and S-1 and S-3 dimers. However, as before the gel system did not give a clear picture of the subunits involved in each dimer.

As a final attempt at a two dimensional gel electrophoresis system, electroelution

**FIGURE 6.5**

**SECOND DIMENSION 17.5% (w/v) ACRYLAMIDE SDS  
POLYACRYLAMIDE GEL SHOWING SEPARATION OF  
SUBUNITS S-1, S-2, S-3, S-4 AND S-5 AND CROSS-LINKED  
AGGREGATES.**

**THE GEL SHOWS THAT NONE OF THE CROSS-LINKED  
AGGREGATES WERE REDUCED INTO THEIR NOMOMERIC  
SUBUNITS FOLLOWING SOAKING IN A REDUCING  
BUFFER.**

x-links

S-1  
S-2  
S-3

S-4/S-5



**FIGURE 6.6**

**SECOND DIMENSION 17.5% (w/v) ACRYLAMIDE SDS  
POLYACRYLAMIDE GEL, WITH 10mM DTT WITHIN THE  
STACKING GEL MATRIX.**

**THE GEL SHOWS DIAGONAL SEPARATION OF S-1, S-2, S-3,  
S-4 AND S-5 AND THE REDUCTION OF SOME CROSS-  
LINKED SUBUNITS.**

**THE GEL IS VERY DIFFICULT TO INTERPRET AND POSITIVE  
DETERMINATION OF THE SUBUNITS INVOLVED IN EACH  
LINKAGE WAS NOT POSSIBLE.**

x-links

S-1  
S-2  
S-3

S-4  
S-5

of the cross-linked species from the first dimension gradient was tried. The cross-linked aggregates were again separated on a first dimension 10% - 20% (w/v) acrylamide gradient SDS polyacrylamide gel. A portion of this gel was fixed and silver stained with the bands corresponding to the aggregates of interest being cut from the remainder of the gel. The strips were cut into 1cm x 2mm portions and the proteins electroeluted from the gel using an ISCO apparatus. The buffer used for electroelution was 10 mM potassium phosphate, pH 7.5, 1 M urea and a current of 30 mA for 4 hours was applied to the system.

The concentrated aggregates were reduced with 0.1% (v/v) mercaptoethanol and boiled in 0.1% (w/v) SDS for 5 minutes before being applied to a second dimension. This method again proved unsuccessful as insufficient protein was eluted from the first dimension to produce a conclusive pattern of monomeric subunits within the second dimension. The problem of the electrophoretic mobilities of the individual subunits under reducing conditions also further complicated analysis of the second dimension gels. Further to this, once sufficient protein had been concentrated from a number of first dimension gels, the silver stained second dimension gels were poorly resolved, with very high background staining, again giving no conclusive results. It was obvious that an alternative approach was necessary to confirm the predictions, based on molecular mass analysis, of which subunits are present in some of the dimers.

#### 6.4.2. Western blotting

Cross-linked species were again separated on a 10% - 20% (w/v) acrylamide, SDS polyacrylamide gel. Strips from these gels were removed and the proteins transferred onto nitrocellulose by the method of Towbin *et al* (1978). The antibodies used were anti-peptide antibodies raised against synthetic peptides derived from the amino acid sequence of either S-1, S-2 or S-3 by Richard Seabrook at CAMR, Porton Down (Seabrook, *et al* 1990). Horseradish peroxidase conjugated anti-mouse IgG was the second antibody and the bands were visualised by 4-chloro,2-naphthol and hydrogen peroxide. The blotting results are shown in Figure 6.7. These confirm the results previously proposed with the DSP cross-linker (insufficient antibody being

**FIGURE 6.7**

**WESTERN BLOTTING ANALYSIS OF DSP CROSS-LINKED  
SUBUNITS.**

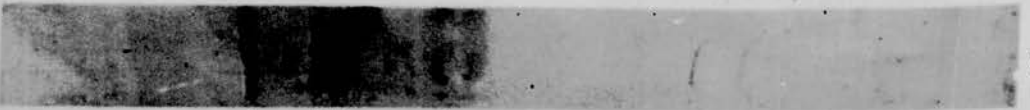
STRIP 1.	BLOT WITH	S-1 ANTIBODY
	BAND 1	S-1/S-2 DIMER
	BAND 2	S-1/S-3 DIMER
	BAND 3	S-1 SUBUNIT
STRIP 2	BLOT WITH	S-2 ANTIBODY
	BAND 1	S-2/S-1 DIMER
	BAND 2	S-2/S-4 DIMER
	BAND 3	S-3/S-4 DIMER
	BAND 4	S-2 SUBUNIT
STRIP 3	BLOT WITH	S-3 ANTIBODY
	BAND 1	S-1/S-3 DIMER
	BAND 2	S-2/S-4 DIMER
	BAND 3	S-3/S-4 DIMER
	BAND 4	S-3 SUBUNIT

STRIPS 2 AND 3 SHOW THAT THE S-2 ANTIBODY CROSS-  
REACTS WITH SUBUNIT S-3 AND THAT THE S-3 ANTIBODY  
CROSS-REACTS WITH SUBUNIT S-2 WHICH IS NOT  
SURPRISING DUE TO THE HIGH DEGREE OF HOMOLOGY  
BETWEEN THE TWO SUBUNITS.



1- 2- 3- 4-

3



1- 2- 3- 4-

2



1- 2- 3-

1

available to Western blot the DST linker strips). Strip one shows the S-1 antibody recognition with subunit S-1 and two cross-linked aggregates being identified. Strip two shows the S-2 results, again showing the S-2 subunit and the recognition of two cross-linked aggregates. It is important to note that one of the S-2 cross-linked bands is the same as one recognised by the S-1 antibody. The final strip is the results from the S-3 blot. Again two aggregates were recognised with one of the bands being at the same position as the second of the S-1 aggregates.

These results confirm the predicted dimer formation between subunits S-1 with both subunits S-2 and S-3 and show that subunit S-4 or S-5 has also formed dimers with subunits S-2 and S-3. On the basis of molecular mass these second dimers are more likely to be S-2/S-4 and S-3/S-4 dimers, but an S-4 antibody blot would be needed to confirm this 100%, and this was not available.

## **6.5. DISCUSSION**

This chapter has discussed an alternative approach in the investigation into the spatial arrangement of pertussis toxin subunits, namely reversible cross-linking reagents, two-dimensional gel electrophoresis and Western blotting.

### **6.5.1 Cross-linking and two-dimensional SDS polyacrylamide gel electrophoresis**

The two cross-linking reagents used; dithiol (succinimidyl propionate) - DSP and disuccinimidyl tartarate, DST, were both specific for primary and secondary aliphatic amines and produced a reproducible pattern of aggregate formation on a first-dimension 10-20% (w/v) acrylamide SDS polyacrylamide gel. The molecular mass of each of the aggregates were calculated by comparison of their mobilities within these gels to the electrophoretic mobilities of a standard set of proteins and also with the electrophoretic mobilities of the individual toxin subunits, whose masses are known precisely from the derived amino acid composition from their DNA sequences. The molecular mass of each aggregate gave a good idea of which subunits were included

within each conjugate. These primary observations obviously needed to be confirmed due to the large number of possible constructs which could be formed, many of which would have similar masses. Confirmation was hoped to result from the second-dimension separation of the regenerated monomeric subunits contained within each of the aggregates. The 12 Å DSP cross-linked aggregates were never sufficiently reduced after soaking gel strips in a reducing buffer to enable the monomeric subunits to be identified within the second-dimension, and incorporation of thiol within the gel matrices caused added complications to the two-dimensional system, as the toxin subunits run differently under reducing conditions.

None of the many attempts at a two-dimensional system produced gels that confirmed the predictions made from the first-dimension gel. Reduction of the internal disulphide bond within DSP was not achieved, a possible explanation for this could be, that the cross-linked subunits formed very tightly associated aggregates, shielding the disulphide bond from attack with reducing reagents thus preventing the regeneration of the monomeric units. The few results which emerged from the second-dimension gels were consistent with those proposed from the first-dimension, but these results were not conclusive.

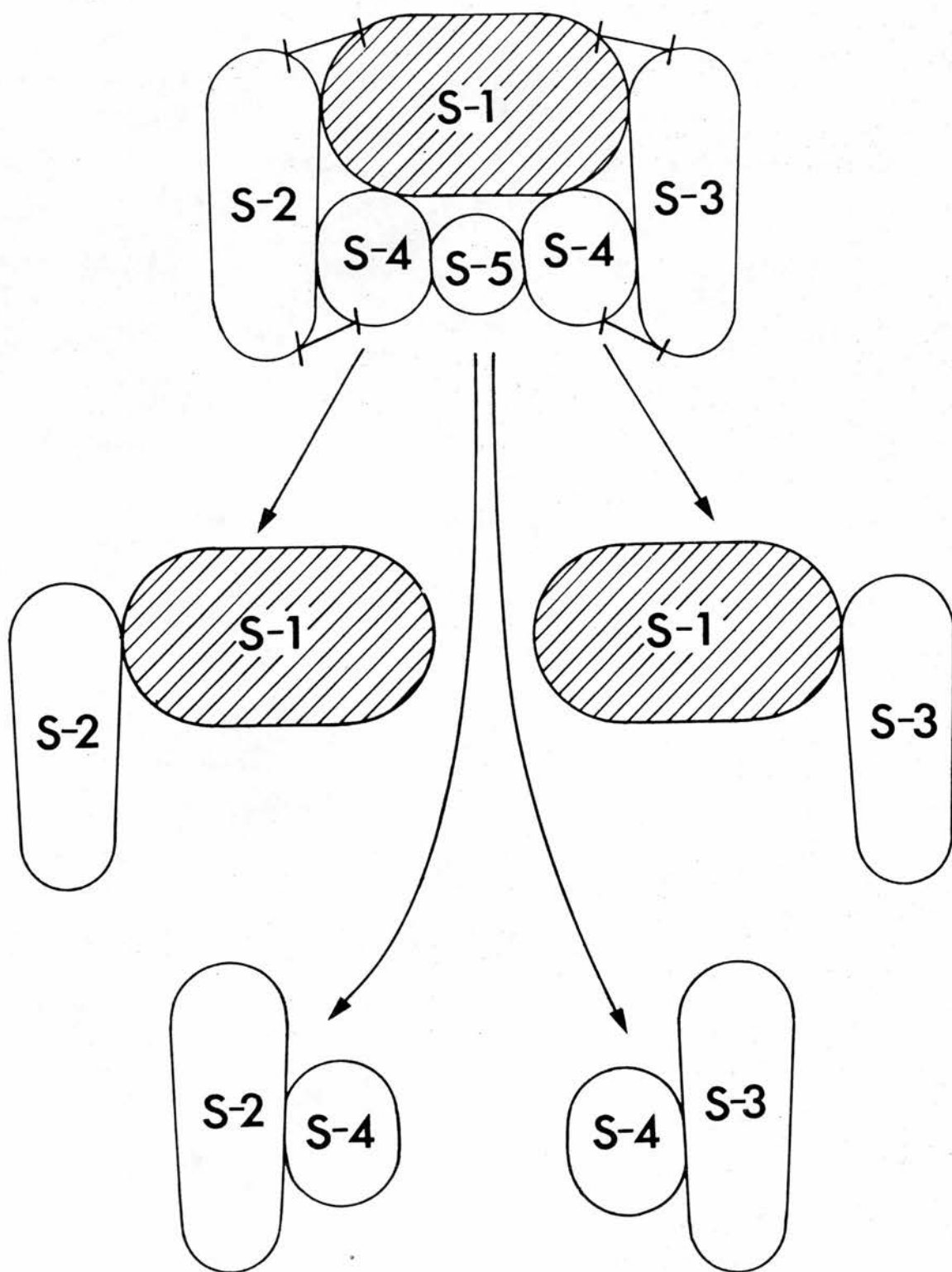
#### **6.5.2. Western blotting**

As the two-dimensional SDS polyacrylamide gel system did not yield conclusive results on the geometry of the individual toxin subunits, an alternative approach for determining the composition of each of the dimers was necessary. Western blotting was the obvious direction to proceed and anti-peptide antibodies against subunits S-1, S-2 and S-3 were employed. These were used to blot nitrocellulose strips of transferred DSP cross-linked subunits. This proved very successful and by comparison with the calculated molecular masses from the first-dimension it was possible to construct a map showing the relative spatial locations of some of the toxin subunits at 12 Å resolution. DSP cross-linked dimers were formed between subunit S-1 with both subunits S-2 and S-3 and between S-4 with both subunits S-2 and S-3. These results are summarised in Figure 6.8. The antibodies did not react with any of the higher molecular mass

**FIGURE 6.8**

**PROPOSED GEOMETRY OF PERTUSSIS TOXIN  
SUBUNITS AT 12Å RESOLUTION**





aggregates, this perhaps being due to their combined conformations preventing recognition by each of the antipeptide antibodies.

The cross-linked results obtained so far provide further evidence for the existence of two dimers, D1 of subunits S-2 and S-4 and D2 of subunits S-3 and S-4, within pertussis toxin (Tamura *et al.*, 1982; Sekura *et al.*, 1983; Rappuoli and Nicosia, 1987). However an S-4 immunoblot would have been useful to confirm this. Based on these results the cross-linking data also suggests that S-1 may sit on a plane, elevated above the B-oligomer subunits in a similar conformation to that previously proposed for cholera toxin (van Heyningen, 1982). S-2 and S-3 may surround S-1 in such a way as to protect it from interaction with the host-cell membrane on binding to and entering into the host cell. No aggregates have, as yet, been identified which involve S-5. This suggests that the other subunits may surround S-5 in such a manner as to protect it from the action of the cross-linkers.

In summary the evidence so far obtained from cross-linking confirms the stoichiometry proposed previously by other workers. There is a great deal more work to be done using cross-linkers, involving those of different lengths and amino acid specificities. Once this task has been undertaken a map showing the precise overall subunit arrangement within the holotoxin should be possible, this geometry being confirmed once the toxins crystal structure has been elucidated.

## CHAPTER 7

### SUMMARY AND FUTURE WORK

The work completed for this thesis has served to characterise pertussis toxin further on the basis of its enzymic activities and overall subunit conformation.

Firstly the toxin's enzymic activity was investigated and a Michaelis constant,  $K_m$  value, of  $(30 \pm 5 \mu\text{M})$  and a  $k_{cat}$  value derived. These values were in close agreement with those already published, (Tamura *et al.*, 1982; Moss and Vaughn, 1984; Burns *et al.*, 1986), when the assay conditions involved 250mM dithiothreitol. It had been previously demonstrated that by increasing the concentration of sulphhydryl reagent within the assay protocol, even to the incredibly high concentration of 250mM, that increasing amounts of nicotinamide were released. This effect was further investigated on the theory that these reagents were acting not only as activating reagents but also as substrates for this enzyme catalysed reaction.

An HPLC system was devised which separated the reaction products and by using radioactively labelled  $^{14}\text{C}$ -cysteine and  $^3\text{H}$ -NAD<sup>+</sup>, the  $^3\text{H}$  label being on the ADP-ribose moiety of this molecule, a reaction product was isolated containing the carbon atoms of the sulphhydryl reagent and the ADP-ribose group of NAD<sup>+</sup>. The following reaction was proposed, with a  $K_m$  of 105mM determined for cysteine in saturating NAD<sup>+</sup> -



The high  $K_m$  value calculated for cysteine illustrates why 250mM dithiothreitol had previously been included within the reaction mixture for maximum nicotinamide production. West *et al.* (1986) have shown that it is a cysteine residue within  $G_i$  which accepts the ADP-ribose moiety from pertussis toxin catalysed ADP-ribosylation, the sulphhydryl group within the NAD<sup>+</sup>-glycohydrolase reaction must be acting in an

analogous fashion.

The binding affinity of  $\text{NAD}^+$  for pertussis toxin was determined using equilibrium dialysis and quenching of the proteins intrinsic fluorescence upon titration with ligand. Equilibrium dialysis involved the direct quantitation of free and bound ligand, and is the simplest method of calculating binding parameters.  $K_d$ , the equilibrium dissociation constant, and  $n$ , the maximum number of moles of substrate bound per mole of protein.  $K_d$  values were obtained at a range of temperatures and an estimate of the enthalpy change on binding of  $\text{NAD}^+$  to the toxin derived. This value of  $30 \text{ kJ mol}^{-1}$  is typical of such an interaction. The number of binding sites on the toxin for  $\text{NAD}^+$  was shown to be one, which was as we would expect as it was unlikely that the toxin should have evolved different binding sites for  $\text{NAD}^+$  in order to catalyse essentially the same reactions of ADP-ribosylation and  $\text{NAD}^+$ -glycohydrolase<sup>activity</sup>.

Fluorescence is an indirect method of determining ligand-binding constants. It utilises the fact that a tryptophan residue within S-1 is involved in the  $\text{NAD}^+$ -binding site and that the intrinsic fluorescence of the protein is reduced on binding of  $\text{NAD}^+$ . The  $K_d$  value derived from this method is in close agreement with that from equilibrium dialysis and both are of the same order of magnitude as the  $K_m$  for the toxin catalysed  $\text{NAD}^+$ -glycohydrolase activity, which is again as we would have assumed.

It would be interesting to repeat the above work using the isolated active subunit in order to show whether its kinetics are the same as those of the holotoxin. In order to do this a suitable system for separating sufficient S-1 must be devised as the protein concentration required for equilibrium dialysis is  $>1.0 \text{ mg/ml}$  to enable accurate calculation of free and bound ligand.

It would also be interesting to investigate the binding affinities of nucleotides, such as ATP and GTP, for pertussis toxin. These ligands have been shown to bind specifically to the toxin and in doing so reduce the affinity of S-1 for the B-oligomer, a step essential for the entry of S-1 within the eukaryotic cell (Burns *et al* 1987; Burns

1988).

The final piece of work undertaken in this project yielded much information on the tertiary structure of pertussis toxin. The two cross-linking reagents used, DSP and DST, demonstrated which subunits were in close association with each other and these results were confirmed using monoclonal antibodies to S-1, S-2 and S-3. The cross-linking data showed that S-1 is in close association with both subunits S-2 and S-3. No cross-linkages were identified between S-1 and the other two subunits showing that they are not accessible to linkage with the reagents used. This suggests that S-1 may be on a plane above the B-subunits, shielding S-4 and S-5, with only S-1 and S-2 being accessible to the cross-linking reagents. S-4 produced dimers with both S-2 and S-3 showing that these subunits are in close association and providing more evidence for the existence of two dimers within the assembled molecule.

Future work on cross-linking would concentrate on DST, the shorter of the two cross-linkers. As this linker is periodate cleavable a reproducible two-dimensional system may be possible as there is no reducing reagent required which would complicate analysis of the results, the toxin subunits running differently in reducing and non-reducing conditions. Once results have been collected from this it is hoped that a three-dimensional map of the spatial localisation of the subunits would be possible. The results from the cross-linking studies will hopefully be confirmed once the crystal structure of the toxin has been deduced. Crystals of the toxin have been grown by Stella Fawcett at Edinburgh and are presently undergoing X-ray analysis on a grant supported by the Medical Research Council.

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## APPENDIX

**A 1** Further binding at 7°C

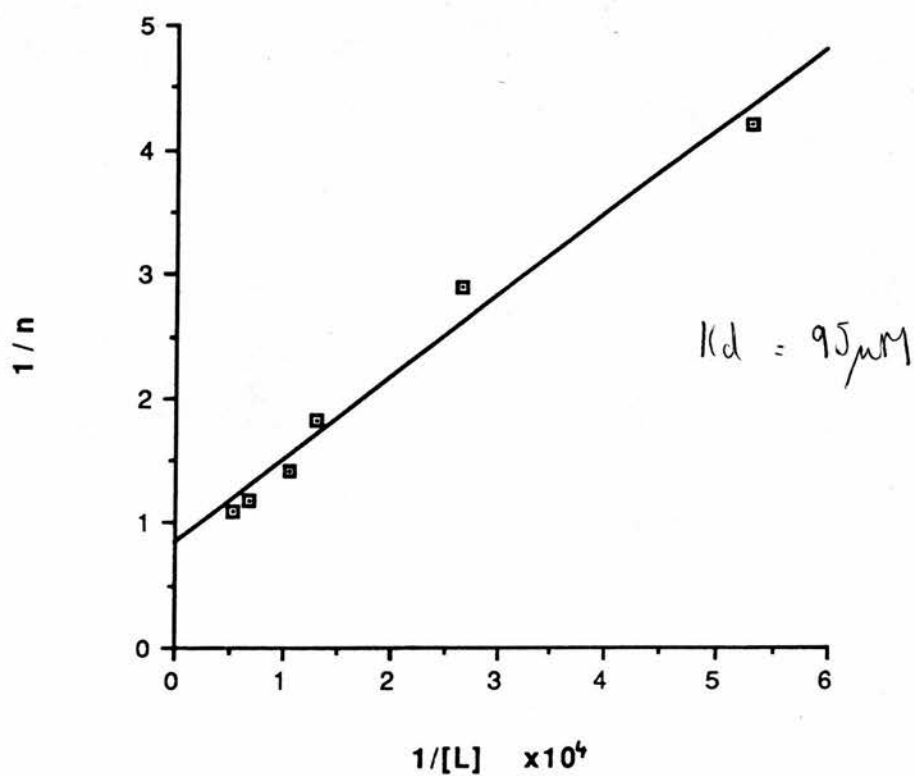
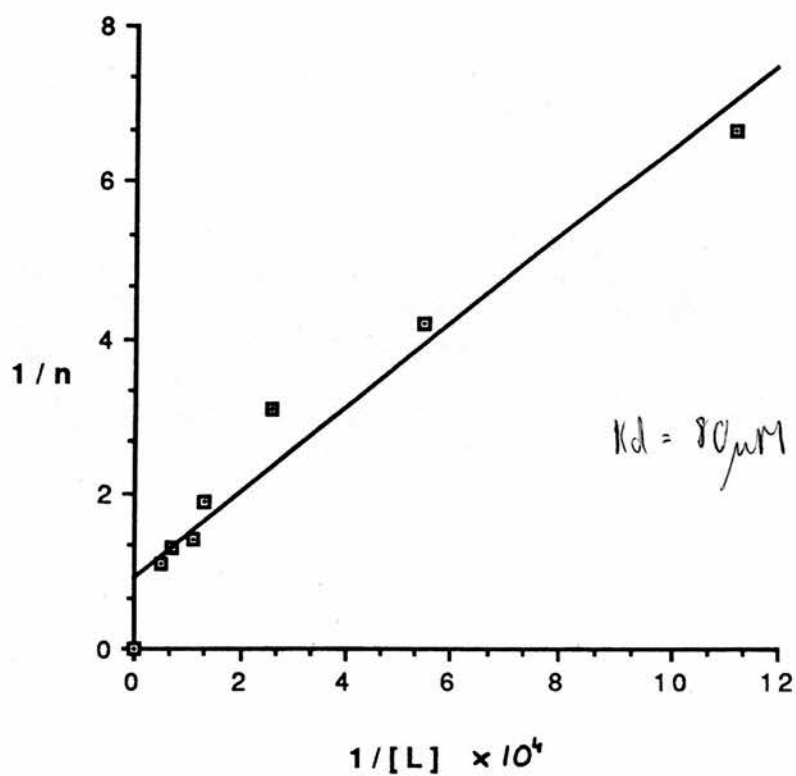
**A 2** Further binding at 20°C

**A 3** Further binding at 23°C

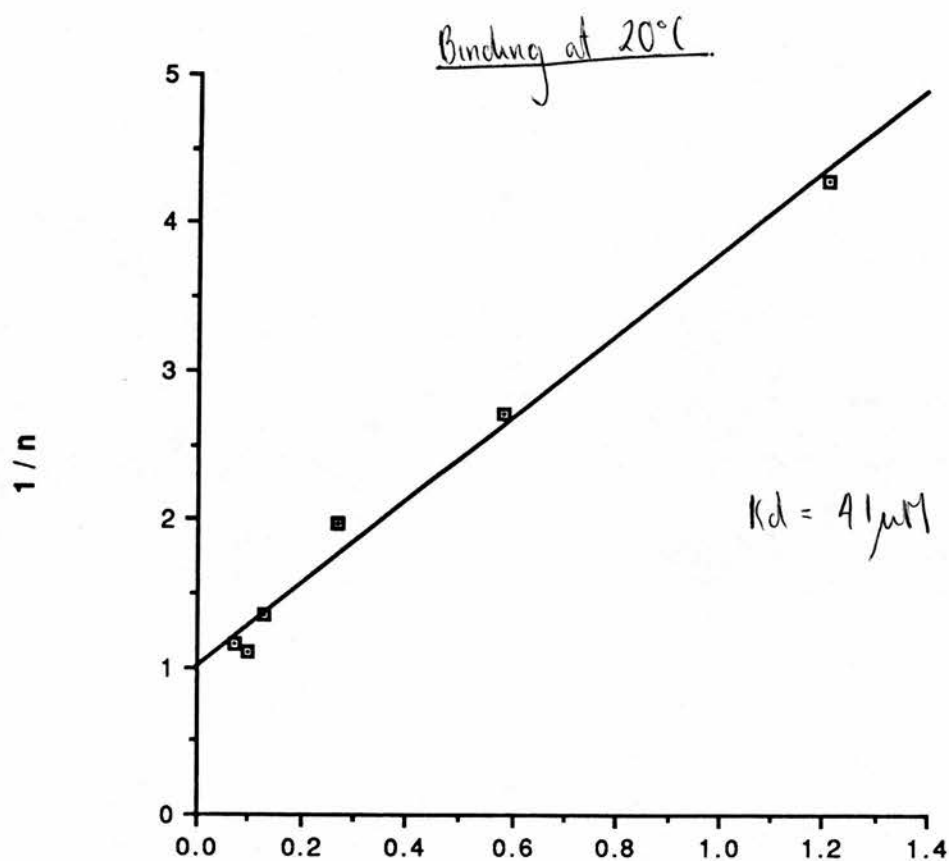
**A 4** Further binding at 30°C

**A 5** Further binding at 40°C

Binding at 7°C



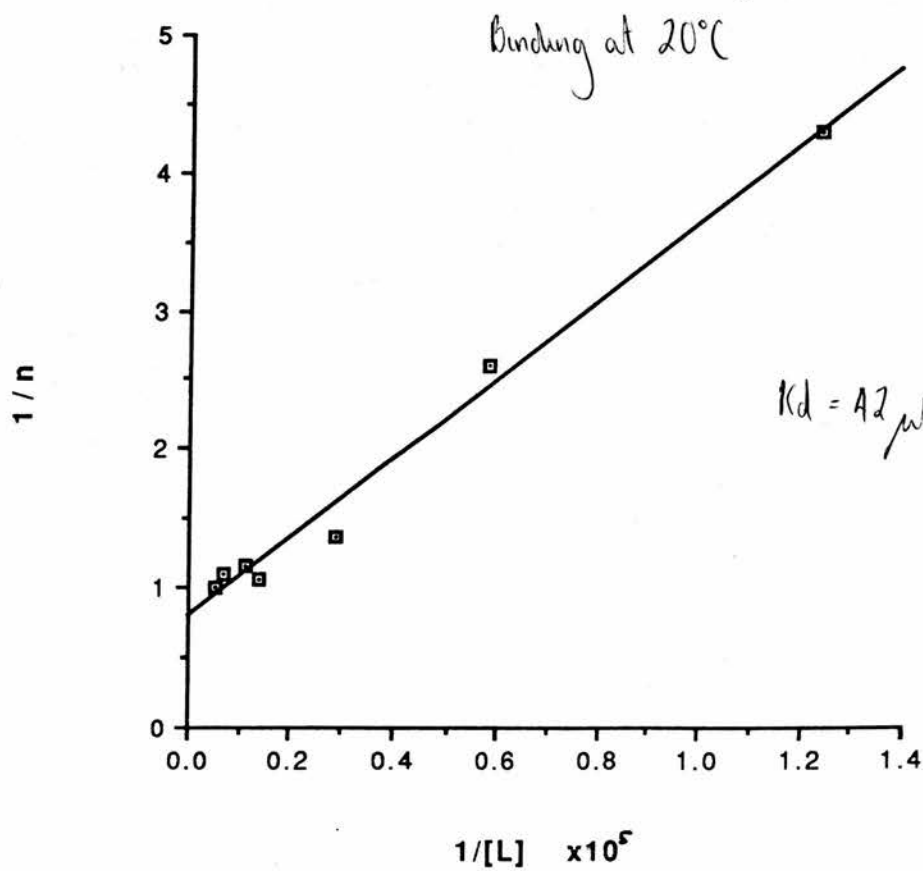
$[L] = [\text{free NAD}^+]$   
 $n = \frac{\text{total moles of NAD}^+ \text{ bound}}{\text{moles of protein}}$



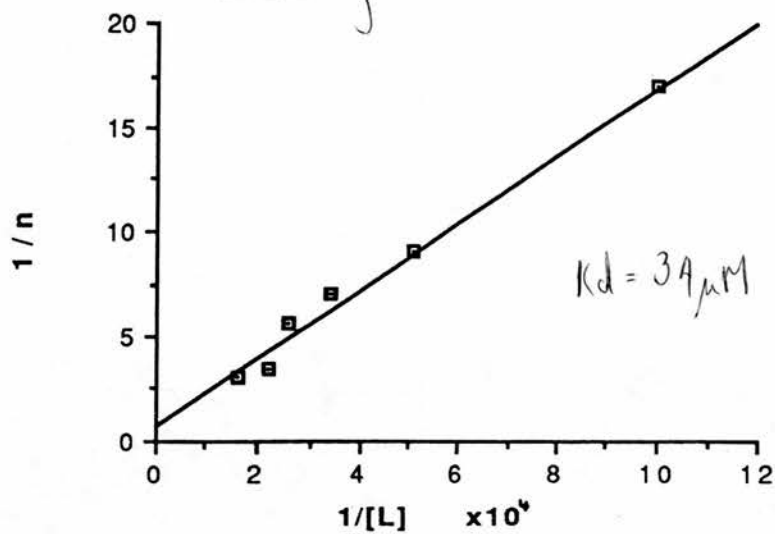
$1/[L] \times 10^5$

$[L] = [\text{free NAD}^+]$

$n = \frac{\text{total moles of NAD}^+ \text{ bound}}{\text{moles of protein}}$



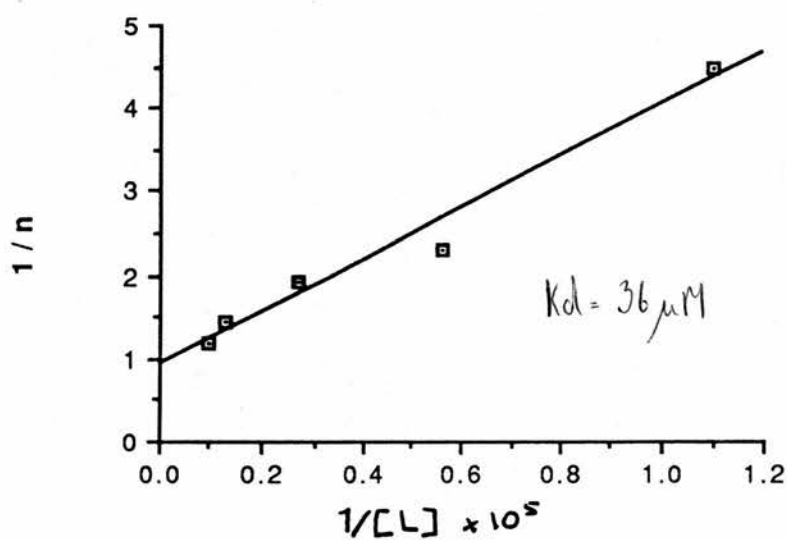
Binding at 23°C



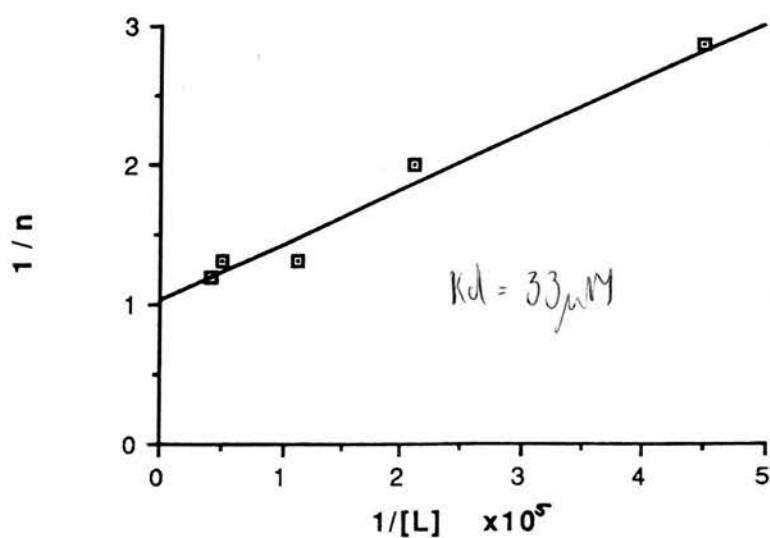
$$K_d = 34 \mu\text{M}$$

$$[L] = [\text{free NAD}^+]$$

$$n = \frac{\text{total sites of NAD}^+ \text{ bound}}{\text{moles of protein}}$$

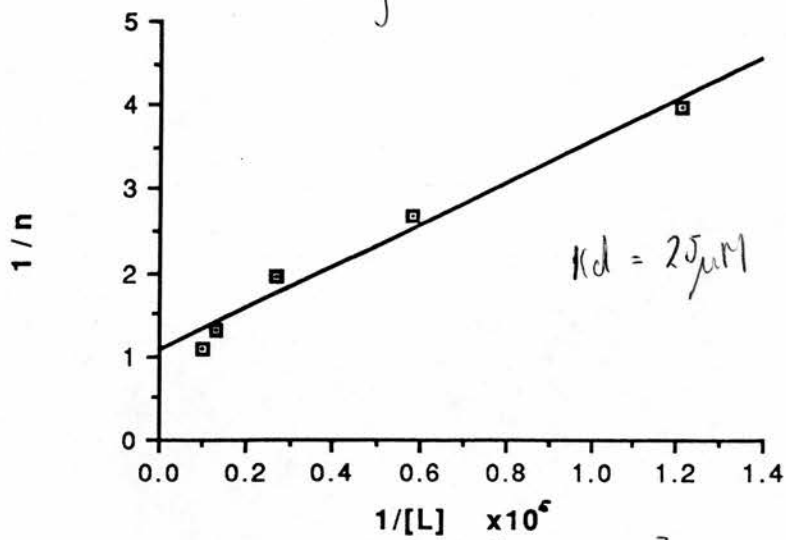


$$K_d = 36 \mu\text{M}$$



$$K_d = 33 \mu\text{M}$$

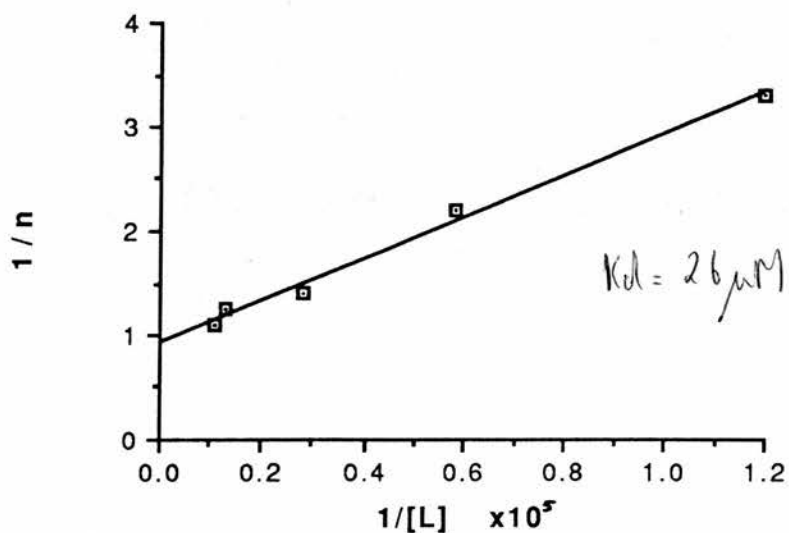
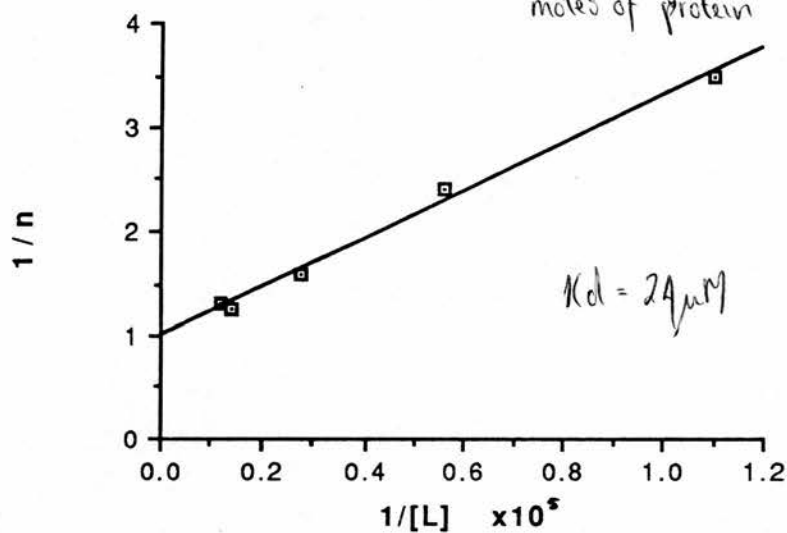
Binding at 30°C



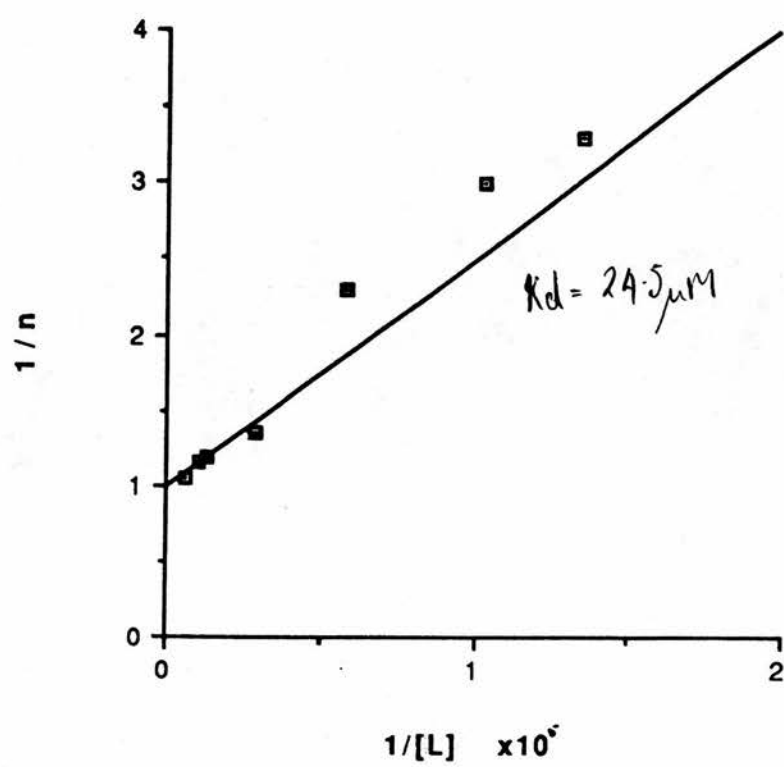
$1/[L] \times 10^5$

$[L] = [\text{free NAD}^+]$

$n = \frac{\text{total moles of NAD}^+ \text{ bound}}{\text{moles of protein}}$

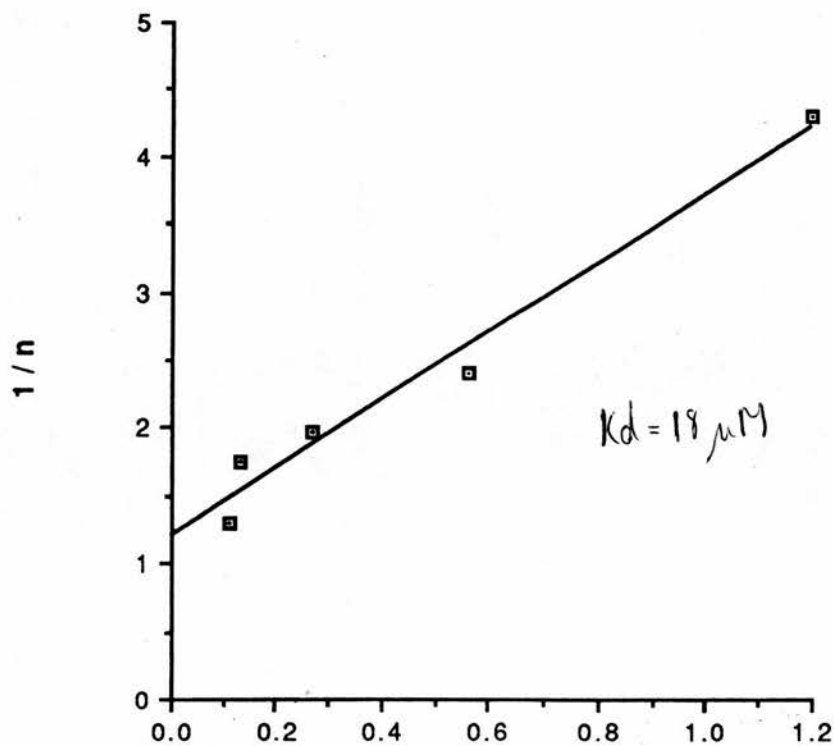






# Binding at 40°C

A5



$$1/[L] \times 10^5$$

$$[L] = [\text{free NAD}^+]$$

$$n = \frac{\text{total } [NAD^+]_{\text{bound}}}{\text{moles of protein}}$$

